Philipps Universität Marburg

Recruitment of erythrocyte membrane components by apicomplexan parasites *Babesia divergens* and *Plasmodium falciparum*

DISSERTATION

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Preetish Gangopadhyay

aus Kalkutta, Indien

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Zweitgutachter: Prof. Dr. Ralf Jacob

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"I slept and dreamt that life was joy.

I awoke and saw that life was service.

I acted and behold, service was joy"

Rabindranath Tagore

To my Ma, Baba, Didi, Sonai, Mom-mom and Ayan

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List of abbreviations

AE1	Anion exchanger 1
AK2	Adenylate Kinase 2
AMA1	Apical membrane antigen 1
AQP/Aqp	Aquaporin
Cy2	Cytochrome 2
Cy3	Cytochorme 3
DBGA	Duffy group binding antigen
DRM	Detergent resistant microdomains
ER	Endoplasmic reticulum
GPA	Glycophorin A
GPI	Glycosyl-phosphatidylinonositol
IMC	Inner membrane
iRBC/IRBC(s)	Infected red blood cell(s)
KAHRP	Knob associated histidine rich protein
MC	Maurer's clefts
MSA	Merozoite specific antigen
MSP1	Merozoite specific protein 1
NPP	Novel permeation pathways
PAGE	Poly acrylamide gel electrophoresis
PBS	Phosphate buffered saline
PEXEL/VTS	Plasmodium export element/Vacuolar targeting signal
PfEMP1	*Plasmodium falciparum* erythrocyte membrane protein 1
PfRh	*Plasmodium falciparum* reticulocyte binding homolog
PIC	Protease inhibitor cocktail
PMSF	Phenylmethylsulphonyl fluoride
PPM	Parasite plasma membrane
PSAC	Plasmodium surface anion channel
PTEX	Plasmodium trasnlocon of exported proteins
PV	Parasitophorous vacuole
PVM	Parasitophorous vacuole membrane
RBC	Red blood cell
RBCM	Red blood cell membrane
RON	Rhoptry neck
SDS	Sodium dodecyl sulphate
SERA	Serine rich antigen
SERP	Serine rich protein
TVM/TVN	Tubovesicular membrane network
VESA1	Variant erythrocyte surface antigen 1
VMSA	Variable merozoite surface antigens

Summary

The fully differentiated mammalian erythrocyte lacks nucleus and in turn lacks biosynthetic machinery. It neither endocytoses nor phagocytoses and is restricted, in terms of nutrient resources available within. Haemoglobin occupies most of its volume and glycolysis provides the energy it needs for metabolic activities. It is enveloped by a single layer of plasma membrane and has no inner membranes. However it has a remarkable structural stability provided by the compact cytoskeleton underlying its plasma membrane and 'docked' to it by the actions of various high copy number and transmembrane proteins. Seemingly, it lacks most of the prerequisites for parasite invasion and growth.

Amongst the intracellular pathogens Apicomplexans are unique in their own way. They actively invade their respective host cells, circumventing host mediated endo/phagocytosis. During the active invasion they form a unique compartment termed 'parasitophorous vacuole' (PV) surrounded by the 'parasitophorous vacuole membrane' (PVM) and stay (if at least temporarily) sequestered within. This in turn helps these pathogens to avoid the lytic environment of the endo-phagosomal compartments of the host. In comparison to the phago-lysosomal compartments, the PV has limited capacity to fuse with host endomembrane system and is capable of retaining the pH at neutral. Additionally the active forms of invasion allow the Apicomplexa to invade and survive within a large array of cells.

There are a few parasitic protozoa that infect erythrocyte and the erythrocyte does not play any essential or even obligatory role in the parasite survival and subsequent development for most of these, but for two apicomplexan parasites of the genera *Plasmodium* and *Babesia.* For these parasites, the host erythrocyte plays a vital role in survival and in associated pathogenicity (directly or indirectly). Moreover these two parasites are also known to alter the host cell differentially; for what seems customising it to specific requirements. Of these alterations one prominent is the formation of the unique vacuolar compartment 'parasitophorus vacuole' surrounded by 'parasitophorus vacuole membrane. However our idea about the contribution of the host cell towards the PVM is limited. In absence of any marker for PVM, the formation and the fate too had not been studied in details but in a few apicomplexa.

I took the advantage of a *Babesia divergens* strain, adapted to human erythrocytes (*B. divergens* normally infect cattle or immuno-suppressed humans) and used *Plasmodium falciparum* clone

3D7 (routinely cultured in human erythrocyte) and did a detailed comparative analysis between the PVM formed the invasion of these two related obligate intracellular apicomplexa in identical host cell (erythrocyte).

Ultrastructure analysis of infected erythrocytes revealed that unlike *Plasmodium falciparum*, which remained inside the PV all along the intra-erythrocytic development, the *Babesia divergens* lost its PVM sometime soon after invasion: an observation, possibly indicating that the maintenance of the PV *per se* as a protective environment is not a prerequisite for this parasite growth.

Thereafter with a strict selection of erythrocyte membrane proteins (membrane anchor containing, membrane spanning, cytoskeletal, cytoskeleton associated and erythrocyte surface receptor), reportedly internalized or discounted during the invasion by *P. falciparum* and present or absent on the newly formed PVM based on their association with erythrocyte cytoskeleton, I performed epifluorescence microscopy and biochemical analysis. I aimed to demonstrate the fate of these proteins parallaly in *P. falciparum* and *B. divergens* infected erythrocytes. I took help of immuno-electron microscopy to confirm my results.

With fluorescence microscopy, I could show that both of the parasites took up labelled lipid components were from the labelled erythrocyte surface and recruited them onto their respective PVMs. However there was a difference in the recruitment of proteins between these two. A high copy number, erythrocyte membrane protein (Band 3) and a cytoskeletal protein (Spectrin) was found present in the PVM of *Babesia* divergens but not in PVM of *Plasmodium falciparum*. Parallel to this in *B. divergens* infected erythrocytes; PVM-localization could not be confirmed for few proteins, for which incorporation into the PVM of *P. falciparum* had been suggested in several reports.

Altogether the results obtained from this study suggest that the recruitment or exclusion of specific membrane components is determined in a parasite specific manner and is not regulated by the intrinsic properties of the erythrocyte membrane. However incorporation or exclusion of different proteins may also reflect difference in the preferred entry sites for these parasites, leading ultimately to the difference in components of the PVM and show the possibility of using such parasites as molecular tools for understanding the inducible physiological processes, generally silent in such quiescent cells (erythrocyte).

Zusammenfassung

Der ausdifferenzierte Säugerzellen-Erythrozyt besitzt keinen Zellkern und daher auch keine Biosynthese-Maschinerie. Er betreibt weder Endo- noch Phagozytose und ist daher auf Nähourstoffe im Zellinneren angewiesen. Hämoglobin macht den größten Teil des Volumens aus, die für metabolische Aktivität benötigte Energie wird durch Glykolyse gewonnen. Der Erythrozyt besitzt eine einzelne Plasmamembran ohne innere Membranen. Durch das kompakte Zytoskelett unter der Plasmamembran, das durch verschiedene Transmembranproteine verankert ist, erreichen Erythrozyten jedoch eine bemerkenswerte Stabilität. Auf den ersten Blick fehlen die meisten Voraussetzungen für eine Invasion durch Parasiten.

Unter den intrazellulären Parasiten nehmen die Apicomplexa eine besondere Stellung ein. Sie invadieren ihre jeweilige Wirtszelle aktiv und umgehen so Endo- oder Phagozytose durch den Wirt. Während der aktiven Invasion bilden sie ein einzigartiges Zellkompartiment aus, die parasitophore Vakuole (PV), die von der parasitophoren Vakuolenmembran (PVM) umgeben ist. In diesem Zellkompartiment verbleiben die Parasiten, zumindest zeitweilig. Dies wiederum hilft den Pathogenen, der lytischen Umgebung des endophagosomalen Kompartiments der Wirtszelle zu entgehen. Im Vergleich mit dem phagolysosomalen System hat die PV nur begrenzte Kapazität, mit der Zellmembran der Wirtszelle zu fusionieren. Somit kann der pH-Wert neutral gehalten werden. Des Weiteren erlaubt die aktive Invasion der Apicomplexa, ein breites Spektrum an Zellen zu befallen und in ihnen zu überleben.

Es gibt nur wenige parasitische Protozoen, die Erythrozyten befallen. Außer für die apicomplexen Parasiten der Gattungen *Plasmodium* und *Babesia* spielen Erythrozyten keine wichtige oder obligate Rolle für intrazelluläre Parasiten. Für die beiden genannten Parasiten jedoch spielt der Erythrozyt eine wichtige Rolle, sowohl für das Überleben als auch für die Pathogenität (direkt oder indirekt). Es ist bekannt, dass diese Parasiten die Erythrozyten auf verschiedene Art und Weise verändern, um sie an ihre Bedürfnisse anzupassen. Die prominenteste Veränderung ist die Bildung der parasitophoren Vakuole, die von der parasitophoren Vakuolenmembran umgeben ist. Wie genau die Wirtszelle zur Bildung der PVM beiträgt ist jedoch bisher unbekannt. Ohne Marker für die PVM wurde bisher, mit der Ausnahme weniger Apicomplexa, weder die Bildung noch das Schicksal der PVM untersucht.

Für diese Arbeit habe ich einen an humane Erythrozyten angepassten Stamm von *Babesia divergens* (*B.divergens* befällt normalerweise Rinder oder immunsupprimierte Menschen) und den *Plasmodium falciparum*-Stamm 3D7 (standartmäßig in humanen Erythrozyten kultiviert) benutzt und eine detaillierte vergleichende Analyse zwischen der PVM, die während der Invasion von diesen verwandten, obligat intrazellulären, Apicomplexa in identischen Wirtszellen (Erythrozyten) gebildet wird, durchgeführt.

Durch Ultrastrukturanalysen konnte gezeigt werden, dass im Gegensatz zu *Plasmodium falciparum*, welche während der gesamten erythrozytären Entwicklung in der PV verbleibt, *Babesia divergens* die PV bald nach der Invasion verliert. Diese Beobachtung könnte darauf hinweisen, dass die Instandhaltung der PV *per se* als schützende Umgebung keine Grundvoraussetzung für Parasitenwachstum ist.

Danach wurde mit strikter Auswahl an erythrozytären Membranproteinen (Proteine, die Membrananker enthalten, membranspannend, Teil des Zytoskeletts, mit dem Zytoskelett assoziiert oder Oberflächenrezeptoren der Erythrozyten sind), die bekannter Weise während der Invasion von *Plasmodium falciparum* aufgenommen oder ausgeschlossen werden und auf der neugeformten PVM an- oder abwesend sind (basierend auf der Assoziation mit dem Zytoskelett) Epifluoreszenzmikroskopie und biochemische Analysen durchgeführt. Ziel war es, das Schicksal dieser Proteine parallel in mit *P. falciparum* beziehungsweise *B. divergens* infizierten Erythrozyten zu zeigen. Mit Hilfe von Elektronenmikroskopie habe ich meine Ergebnisse bestätigt.

Mit Fluoreszenzmikroskopie konnte ich zeigen, dass beide Parasiten markierte Lipidbestandteile von der markierten Erythrozytenoberfläche aufgenommen und in die neu gebildete PVM eingebaut haben. Es gab jedoch einen Unterschied in der Rekrutierung zwischen beiden Parasiten. Eine hohe Anzahl an erythrozytärem Membranprotein (Band3) und Zytoskelettprotein (Spektrin) wurde in der PVM von *Babesi divergens* gefunden, jedoch nicht in der PVM von *Plasmodium falciparum.* Parallel dazu wurden in *B. divergens*-infizierten Erythrozyten manche Proteine nicht in der PVM gefunden, für die mehrfach eine PVM-Lokalisierung in *P. falciparum* berichtet wurde.

Insgesamt deuten die Ergebnisse dieser Arbeit darauf hin, dass die Rekrutierung bzw. der Ausschluss von spezifischen Membrankomponenten vom Parasiten gesteuert und nicht durch

intrinsische Eigenschaften der Erythrozytenmembran bestimmt wird. Die Aufnahme oder der Ausschluss von verschiedenen Proteinen könnte auch Unterschiede in der bevorzugten Eintrittsstelle dieser beiden Parasiten widerspiegeln, was letztendlich zu den unterschiedlichen Komponenten in der PVM führt.

1 Introduction

1.1 Intracellular parasitism

Intracellular parasitism supports the parasites development in two distinct ways, by a) protecting the parasite from the host cell immune responses and b) additionally providing it with a nutritious environment to grow in. Hence, it is quite rightly a "life in safe haven" for such parasites [Reviewed by (Sinai and Joiner, 1997)]. A niche like this renders most antibodies-directed against the parasites, ineffective and additionally, allows these to maintain metabolic activities, at a minimal level (Nyalwidhe et al., 2003). In order to reach to and sustain in these desired niches, these parasites seemingly have co-evolved with their hosts and have developed unique mechanisms, to exploit host cellular machinery both to access the host and/or to modify it suitably (Roy and Mocarski, 2007).

Unlike viruses, the eukaryotic and prokaryotic pathogens remain as cellular entities during the host cell invasion and also during their differentiating and multiplication. These pathogens are internalized by their respective host cells as entire cells, and thereafter, to maintain the cellular entities, these pathogens remain secluded (at least initially) in a phagosome or phagosome like compartment. Parasitic protozoa (e.g. *Trypanosoma cruzi*) and bacteria (e.g. *Shigella*, *Rickettsia*) can also be found to be living free inside the host cell cytosol but these are presumably their secondary locations; after the disintegration of or escape from their early endocytic compartments (Sansonetti, 2001, Cossart and Sansonetti, 2004, Ley et al., 1990, Schroeder and Hilbi, 2008).

A broad range of parasites choose phagocytic cells as hosts and this is mostly because of the phagocytic properties of these cells allow passive cell entry of the parasite, eliminating the need of any special entry mechanism on the parasites part (Haas, 2009). Furthermore the parasite containing phagosomes are often taken to the host endosomal network and after progressive development, these fuse with the lysosomes to obtain nutrition (van der Goot and Gruenberg, 2006). Macrophages are a well known phagocyte of the immune system and are also a major antigen presenting cell; serving to link the adaptive and innate immunity. Macrophages are often the hosts of choice for the intracellular parasites like *Coxiella* (Peters et al., 1995) and *Leishmania* (Mosser and Brittingham, 1997).

Use of the host phagosomal network for invasion, more often than not, leads the invading pathogens to the more or less lytic environment of host endosomal networks. A group of protists classified under the monophyletic group of Apicomplexa, however are known to circumvent the phagocytic uptake and in turn resist any contact with the lytic endosomal environment of the host. Most of the members of this group are obligate intracellular parasites and are associated with several diseases in humans and free living animals (Plattner and Soldati-Favre, 2008). The Apicomplexans can induce an alternative mode of host cell entry, driven by the parasite itself (Soldati et al., 2004, Keeley and Soldati, 2004). They utilize the synchronised discharges of their Apicomplexan-specific compartments (located at, what presumably is the apical end of these parasites) (Scholtyseck and Mehlhorn, 1970) and invade the target cells, by a parasite regulated actin-myosin motor driven process (Soldati et al., 2004, Keeley and Soldati, 2004) .

Discharges from the Apicomplexan-specific compartments in these parasites are concomitant with the host cell invasion and presumably, these discharges induce and contribute towards the formation of an unusual vacuolar compartment. In distinction to the endogenous phagosomal compartments, this compartment is duly termed as the 'parasitophorous vacuole' (PV) (Scholtyseck and Piekarski, 1965). PV differs from the host phagosomal compartments in: a) its limited ability to fuse with the host cells endo-membrane network and b) in its ability to maintain the pH close to neutral [Review by (Lingelbach and Joiner, 1998)].

Though the receptor-ligand mediated initial attachment, preceding the active invasion of the host cells, had been characterised for few of the apicomplexan, neither the molecular mechanisms underlying the formation of the PV nor the macromolecular constituents (representing the parasite and/or the host cell) of the PV are well understood.

1.1.1 A brief introduction to Apicomplexa

The extremely large and diverse phylum of Apicomplexa (≥5000 species) belongs to a monophyletic group composed almost entirely of obligate intracellular parasites (Levine and Ivens, 1988). The members affect life in terms of human health (e.g. *Toxoplasma*, *Plasmodium*), veterinary medicine and agriculture (*Babesia*, *Theileria* and *Eimeria*) (Plattner and Soldati-Favre, 2008).

The Apicomplexa along ciliates and dinoflagellates, form the higher order group of Alveolata; a group characterised by the presence of a cortical alveolar system of flattened Golgi apparatus, beneath the plasma membrane (Adl et al., 2005). In Apicomplexa it is often referred to as Inner membrane complex (IMC) (Porchet-Hennere and Nicolas, 1983).

Like many other Dinoflagellets, the Apicomplexa too have retained a remnant chloroplast termed as the Apicoplast (Gardner et al., 2005). However the defining characteristic of this group is the set of sub-microscopic organelle termed as the apical organelles localized at the anteriority of the parasites and are involved presumably in mediating the host cell adhesion and subsequent invasion (Sibley, 2011).

1.1.1.1 Morphology of Apicomplexans

Most of the Apicomplexans have a complex life cycle, involving transmission cycles between and within hosts mediated by the help of invasive stages are broadly termed as zoites. The zoites are characterised by the presence of three sets of secretory compartments, termed together as the apical organelle (Aikawa, 1971, Scholtyseck and Mehlhorn, 1970) and these show gliding motility (Soldati et al., 2004).The apical organelle secretes substances enabling the zoites to adhere to the host cell surface and mediate invasion. Of the three apical organelles, the dense granules release their contents on the zoite's surface prior to adhesion, whereas the other two named microneme and rhoptries, release their contents apically during the invasion (Lingelbach and Joiner, 1998, Preiser et al., 2000, Perkins, 1992, Carruthers and Sibley, 1997). Post invasion, the substances secreted from the apical organelle lead to modifications of the intracellular environment and also play role in parasite egress (Bannister and Mitchell, 2003).

The club shaped Rhoptries (derive their name from the Greek letter rho) are the largest amongst the 3 apical organelles. The size and number of the rhoptries vary amongst the zoites of different genera of Apicomplexa and some correlation seem to exist between the size of the rhoptries and the size of zoites; where the larger the zoite is the larger its rhoprty is. Size and number of the rhoptries are seemingly important from the perspective of invasion and in the formation of an optimally sized, vacuolar compartment to seclude zoite inside after invasion (Margos et al., 2004). Rhoptries occupy approximately 10-30% of the total cell volume of the zoites and are packed with secretory granular materials (Bannister and Mitchell, 2003). Distributed between the bulbous part (Rhoptry proteins ROPs) and/or the neck (Rhoptry neck protein, RONs), the

rhoptries harbour up to 30 different proteins; of which some are kinases, essentially involved in the pathogenicity (Bannister and Mitchell, 2003, Bannister and Mitchell, 1989, Ndengele et al., 1995, Sam-Yellowe et al., 1995, Crewther et al., 1990).Rhoptries collapse and change their appearance once the contents are discharged during the invasion and membranous structures become apparent (Bannister and Dluzewski, 1990).

In *Plasmodium*, several individual observations like a) fusion of *Plasmodium* spp. transport vesicles to the rhoptry compartments (Bannister and Mitchell, 1995), b) presence of a typical N-terminal signal sequence in rhoptry proteins guiding translocation across the ER membrane [Reviewed by (Lingelbach, 1993)] and c) blockage of post translational proteolytic modification of rhoptry proteins by BFA (Ogun and Holder, 1994) have lead to the idea of possible involvement of these rhoptries in the classical secretory pathway. However the external signal or the signalling mechanisms leading to the release of the rhoptry proteins of Apicomplexa are not yet clear

Micronemes are 'cigar shaped' tiny apical organelle, densely packed with granular content and are involved in the recognition and subsequent adhesion process to the host cell (Lingelbach and Joiner, 1998, Mercier et al., 2005).These can secrete large amount of proteins onto the zoite's surface and in turn assist in its motility. Compared to Rhoptries these are relatively smaller in size but are present in larger numbers per cells (Tomley and Soldati, 2001). In Apicomplexa, number of micronemes per cell varies depending upon the developmental stages of the parasite, the genus and the species (Tomley and Soldati, 2001, Tomley, 2009, Carruthers and Tomley, 2008).

Some of the micronemal proteins have linear hydrophobic stretches presumably involved in and as transmembrane domains; whereas the marker proteins of the micronemes reportedly contain N-terminal signal sequence- suggesting their ultimate target to be the ER (Adams et al., 1990, Sim et al., 1990, Galinski et al., 1992, Fourmaux et al., 1996, Wan et al., 1997). Though the trigger and the triggering mechanism behind the secretion of micronemal proteins (leading to the initial contact) is not much known about but it has been shown that the secretion is upregulated upon contact with the host cell (Carruthers and Sibley, 1997). Extensive numbers of homologous domains are found present between the micronemal secretions of different Apicomplexa and this

supports the hypothesis of a commonality amongst molecular mechanism underlying host cell recognition, attachment and subsequent invasion by these parasites (Tomley and Soldati, 2001).

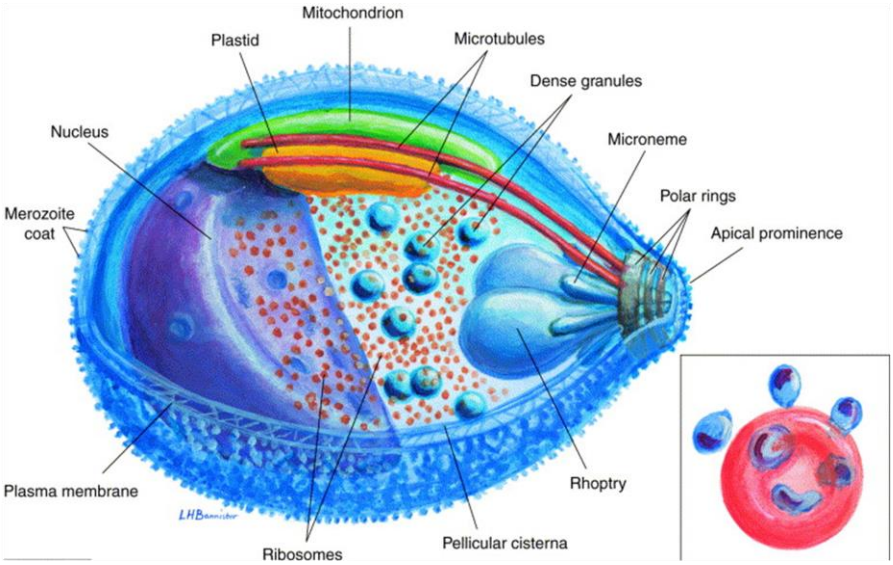


Figure 1.1 3-D reconstruction of the merozoite of *Plasmodium falciparum* showing the secretory apical organelles [Adapted from: A Brief Illustrated Guide to the ultrastructure of *Plasmodium falciparum* Asexual Blood Stages (Bannister et al., 2000)].

The Rhoptries (blue pouch) are secreted one at a time through the Polar (conoid) ring present at the apical tip. The micronemes (in darker blue) are docked at the posterior side of the polar rings at the tip. The Dense granules (in dark blue floating bodies) are secreted through "Open IMC-windows" present beside the apical tip. During the process sub-pellicular microtubules (in red bundles) maintain the polarity and the shape of the cells (Inset: A relative depiction of size and organization of merozoite the merozoite on erythrocyte during invasion)

Dense granules are densely packed membrane bound vesicles, found present in varied number and size amongst the Apicomplexa (Dubremetz et al., 1998) and these resemble the dense matrix granules of mammalian exocrine and neuro-endocrine cells[Reviewed by (Lingelbach and Joiner, 1998)]. The dense granules release their protein contents during or after invasion and these can remain soluble inside the PV or get integrated in the PVM or TVM, presumably for the modification of the PV environment (Mercier et al., 2002, Bannister et al., 1975, Bannister and Dluzewski, 1990). In *Plasmodium* some of the dense granule proteins contain a typical N-terminal signal sequence suggesting their translocation destination to be ER [reviewed by (Lingelbach and Joiner, 1998)].

1.1.1.2 Intracellular life of Apicompexans

Forms of substrate dependant gliding motility concomitant with the release of the proteins from the apical organelles, is the key to the host cell invasion by the Apicomplexan (Menard, 2001) parasites [e.g. *Plasmodium* spp.(Baum et al., 2006), *Babesia* spp.(Asada et al., 2012), *Cryptosporidium* spp. (Wetzel et al., 2005) and *Toxoplasma* spp (Sibley, 2004, Wetzel et al., 2004)]. But unlike seen in most of the apicomplexan parasites, the host cell invasion by *Theileria parva* is a passive form of internalization where after an initial host cell (lymphocyte) recognition and attachment, a zippering mechanism concomitant with the shedding of parasite surface proteins, lead to the internalization of the parasite (Plattner and Soldati-Favre, 2008, Shaw, 2003).

This active form of invasion found in most of the Apicomplexan parasites, assisted with the discharges from the apical organelle, render these parasites an advantage of invading a large spectrum of cells and also to modify them suitably to continue on with their respective infections (Sibley, 2004, Sibley, 2011, Plattner and Soldati-Favre, 2008). The choice of the host cells is diverse for these member protists. *Toxoplasma gondii*, a ubiquitous parasite affects almost 1/3rd of the world population (Tenter et al., 2000).It has the broadest range in terms of host cells and can infect almost all nucleated cells (Plattner and Soldati-Favre, 2008). The *Cryptosporidium* spp. infects mainly the erythrocytes of all type of vertebrates including of humans (all enucleated, but birds) (Sibley, 2004, Plattner and Soldati-Favre, 2008). Haemosporidian *Plasmodium falciparum*, the causative agent of malaria tropica, infects nucleated hepatocytes and then enucleated erythrocytes and completes the asexual and sexual phases of development in these respectively. However a related haemosporidian *Babesia divergens*, can infect erythrocytes directly and grow within (Sibley, 2011, Plattner and Soldati-Favre, 2008).

As characteristic to the Apciomplexan, *Toxoplasma* spp. form a PV during the host cell invasion to complete the entire development within it and their PVM derives lipids and protein components from the parasite and host alike(Mordue et al., 1999, Suss-Toby et al., 1996, Hakansson et al., 2001). However *Thieleria* infecting erythrocytes and lymphocytes, and *Babesia* (infecting erythrocytes), escape from their respective PVs and continue on with a cytoplasmic development without any damage to the parasite (Sibley, 2011, Plattner and Soldati-Favre, 2008). Apicomplexans of genus *Plasmodium* invade erythrocytes as well and form a PV in the

course of invasion; however much unlike *Theileria* and *Babesia*, *Plasmodium*. retains the PV all along the intra-erythrocytic developmental (Plattner and Soldati-Favre, 2008).

Active forms of invasion in Apicomplexa parasites, allow access to a broad range of target cells to these parasites. This coupled with the varied capacity of these parasites to alter the host cells, help most of them maintain their obligate intracellular lives rather successfully. Many of these parasites are often found associated with wide spread infections and the morbidity and mortality associated therewith.

Table 1.1 Apicomplexan parasites infecting humans

Genera	**Transmission**	**Disease**
Babesia	Tick	Babesiosis, zoonotic disease
Cryptosporidium	Faecal-oral	Watery diarrhoea
Cyclospora	Soil	Watery diarrhoea
Isospora	Soil	Watery diarrhoea
Plasmodium	Mosquito	Malaria
Sarcocystis	Prey-predator	Rare infection
Toxoplasma	Feline and definitive hosts	Neurological manifestations

1.2 Human erythrocyte: an unusual cell and an uncommon host

Differentiated mammalian erythrocytes are devoid of nucleus, as the nucleus gets absorbed by the macrophages (Yoshida et al., 2005) and thereafter other cellular organelle are eliminated by autophagy (Mortensen et al., 2010, Kundu et al., 2008). Thus, these cells lack genetic programme to synthesize and also the ability to transport macromolecules like proteins, lipids and carbohydrates (Schrier, 1985, Chasis et al., 1989, Chasis and Mohandas, 2008). Erythrocytes cannot divide and are limited in terms of cell repair capacity (Kabanova et al., 2009). Phagocytosis is absent in matured erythrocytes (Burns and Pollack, 1988) whereas endocytosis decreases along their development from reticulocytes to differentiated erythrocyte stage (Zweig and Singer, 1979). In its predetermined life span of 120 days it derives energy from glycolysis (van Wijk and van Solinge, 2005) and performs its role in gaseous exchange.

It is a single membrane bound compartment and survives remarkable deformation without being fragmented while travelling through some of the narrowest endothelial slits of the body (up to 1/8th of its own diameter) (Mohandas and Gallagher, 2008, An and Mohandas, 2008) and this remarkable deformability and elasticity of the erythrocyte membrane originates from the tripartite action of: a) the unique lipid components of the membrane, b) the compact cytoskeletal support beneath the membrane and c) the docking proteins which anchor the cytoskeleton to the lipid bilayer giving it elasticity and stability (Yu et al., 1973, Mohandas and Gallagher, 2008).

1.2.1 Erythrocyte membrane components: Lipids

Apart from working as the separating margin between the intra and extra-erythrocytic environments, the erythrocyte plasma membrane also acts as of a permeability barrier between the two [Reviewed by (Smith, 1987)]. Of the lipid components of the plasma membrane, the major are the phospholipids and cholesterol (Ways and Hanahan, 1964). The two phospholipid layers are arranged in a way that the hydrophobic fatty acid chains form the core and the polar heads face the exterior, on both the sides of the plasma (outer leaflet) and erythrocyte cytoplasm (inner leaflet) (Ferrell et al., 1985). Cholesterol is equally distributed between these two layers whereas the phospholipid distribution is asymmetrical (Bretscher, 1972, Verkleij et al., 1973). The outer leaflet contains phosphatidylcholine and sphingomyelin whereas the inner leaflet contains phosphatidylserine and phosphatidylethanolamine (Verkleij et al., 1973, Gordesky and Marinetti, 1973).The forces creating this asymmetry are not well understood but any alteration of this asymmetry is reportedly of serious consequences (Smith and Stubbs, 1987).

The fluidity of this complex structure is maintained by maintaining a) the molar ratio of cholesterol and phospholipids, b) the ratio of sphingomyelin and phosphatidylcholine and c) by the degree of saturation of the acyl chains of the phospholipids [Reviewed by (Smith, 1987)].

Like reported in other biological membranes in erythrocyte too (Lingwood and Simons, 2010), the phospholipid bilayer is rich in non-esterified cholesterol, typically associated with sphingolipids, leading to the formation of the structures called ‘rafts’ (Brown and London, 1998b). These ‘rafts’ are sub-micron protein-lipid clusters which are held together by a high level of cholesterol (Brown and London, 1998a, Brown and London, 1998b) and are believed to be involved in the sorting and signalling processes of the cell (Brown and London, 1998a, Simons and Ikonen, 1997).

These are supposedly capable of lateral movement along the membrane plane and also shuttle between the membrane and internal compartments (Nichols et al., 2001).

The lipid bilayer responds to even slight variations in the surface area of the inner and/or outer leaflet and alterations in either of these surface areas are reportedly associated with corresponding structural changes of the erythrocyte (Ferrell et al., 1985, Lange and Steck, 1984). In absence of *de novo* lipid synthesis pathways in the erythrocyte, lipid renewal pathways seemingly play major role in maintaining the phospholipid turnover of the erythrocyte and exchange of cholesterol between plasma and membrane components maintain the cholesterol contents of the erythrocyte membranes (Shohet et al., 1968, Mohandas and Gallagher, 2008).

1.2.2 Erythrocyte membrane components: Proteins

1.2.2.1 Integral membrane proteins:

1.2.2.1.1 Band 3

Band 3 or Anion exchanger 1 (AE1) is a major integral protein of the erythrocyte membrane and with a very high copy number per cell; it represents 25% of the membrane protein pool by itself (Bruce et al., 2003). Band 3 is composed of 3 dissimilar and functionally distinct domains: a) the hydrophilic, cytoplasmic domain reportedly interacting with peripheral membrane proteins and cytoplasmic proteins alike, b) The hydrophobic transmembrane domain, consisting of multiple membrane- spanning domains forming the anion transporter unit and c) the acidic C-terminal, known to be interacting with carbonic anhydrase (Mohandas and Gallagher, 2008)

The two major functions of Band 3 are: a) transmembrane anion transport, resulting in one-for-one exchange of Cl^- for $HCO3^-$ and b) structural support to the membrane by forming physical link between the lipid bilayer and underlying membrane cytoskeleton (Weed, 1970). This is achieved primarily through its interaction with Ankyrin and protein 4.2 and secondarily through binding to the protein 4.1 and Adducin (Bruce et al., 2003). Such an association is advantageous in preventing any loss of membrane surface (Bennett, 1989, Bruce et al., 2003, Cohen and Foley, 1984).

1.2.2.1.2 Glycophorins

In humans' four unique polypeptides, all representing sialic-acid rich glycoproteins make up the group of Glycophorins. Glycophorins (A, B, C and D) represent 2% of the total membrane

proteins of the erythrocyte (Chasis and Mohandas, 1992). The Glycophorins consist of three domains: a) a heavily glycosylated extracellular domain, b) a hydrophobic domain, forming a single alpha helix and spanning across the bilayer and c) a cytoplasmic domain, containing clusters of basic residues, placed in opposition to the plasma membrane (Chasis and Mohandas, 1992).

The carbohydrate residues present on these glycoproteins impart a net negative charge on the erythrocyte and thus reducing any hitherto interaction (Chasis and Mohandas, 1992). Glycophorin C is o interact with protein 4.1 and p55 to regulate their representation in the membrane (Chasis and Mohandas, 1992). Moreover Glycophorins are known to be involved as receptors for *P. falciparum* invasion (Chishti et al., 1996) and also in *B. divergens* invasion (Cursino-Santos et al., 2014b).

1.2.2.1.3 Aquaporins

Aquaporins belong to a family of major intrinsic proteins and are essentially involved in formation of membrane channel and pores for maintaining an osmotic equilibrium inside the erythrocyte (Agre, 2006, Lee et al., 1997). Aquaporin-1 and 3 are the well studied amongst the others of this family. Aquaporin-1 is a more classical of Aquaporins involved in the formation of water selective pores whereas Aquaporin-3 is more of an aqua-glyceroporin, with moderate permeability for water but higher permeability for glycerol (Agre, 2006, Lee et al., 1997, Roudier et al., 2002).

1.2.2.2 Cytoskeletal protein:

1.2.2.2.1 Spectrin

Spectrin is a 106 amino acid protein consisting of triple helical domains linked by flexible hinge region and with about 200,000 copies per cell, it represents 50-75% of the cytoskeletal protein pool (Yu et al., 1973).

Spectrin is organised as α, β-heterodimers consisting of a 240kDa α-chain and 220kDa β-chains respectively (Hsu et al., 1979, Marchesi, 1979). Each of these chains are composed of multiple homolog of the 106-residue ‘repeats’, folding in, to form the triple alpha helical bundle with the 1st and 3rd helices running parallaly and the 2nd one running antiparallely (Speicher et al., 1992). The spectrin heterodimers self-associate in this typical anti-parallel fashion starting from a dimer

nucleation site which eventually forms the amino terminus of the β-spectrin and the carboxy terminus of the α-spectrin (Ursitti et al., 1991). The rest of the heterodimer follows by a zipper like side by side association and forming a super-coiled wire like structure in turn (Speicher et al., 1992).

The β-spectrin has several binding sites for the other proteins of the cytoskeleton and membrane alike. Near the N-terminal region of the β-spectrin there are binding sites for short actin filaments and protein 4.1, leading eventually to the formation of a junctional complex (Bennett, 1989, Gilligan and Bennett, 1993). At this site Glycophorin-C binds to serve as an additional anchor for the membrane lipid bilayer. Near the C-terminus, spectrin binds with ankyrin, which binds also to the cytoplasmic tail of Band 3 for docking the membrane lipid bilayer to the cytoskeleton beneath (Maier et al., 2009, Gallagher and Forget, 1993, Conboy, 1993, Gilligan and Bennett, 1993).

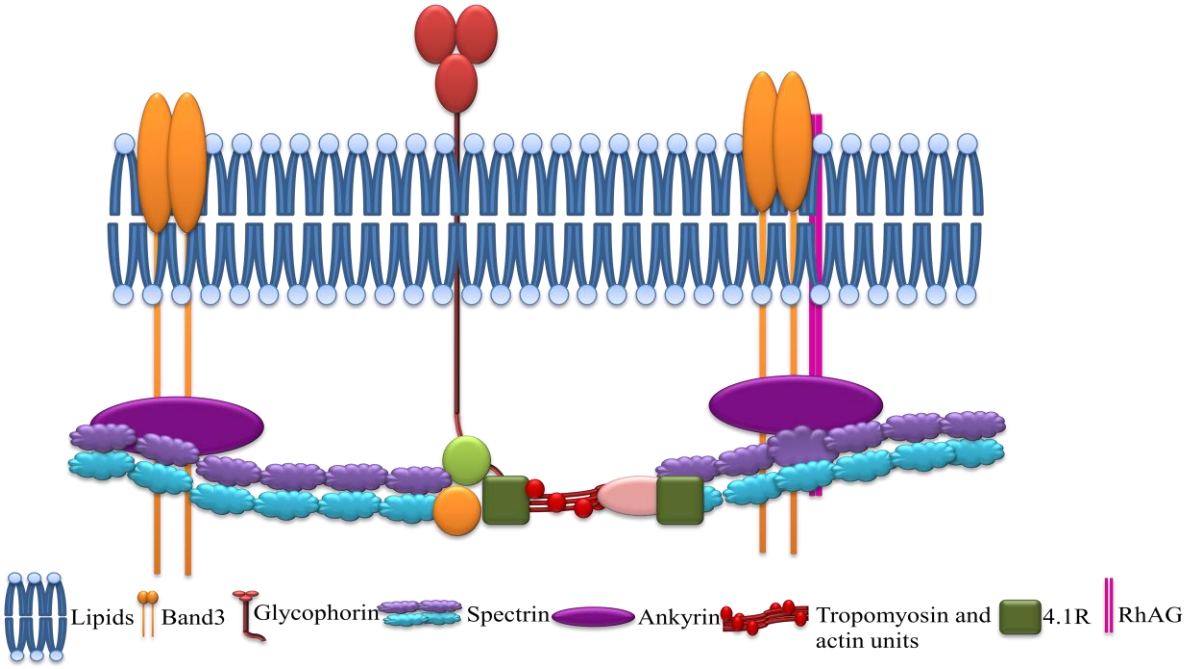


Figure 1.2 organization of the human erythrocyte membrane [Adapted from, Malaria parasite proteins that remodel host erythrocyte. (Maier et al., 2009).

Phospholipids and cholesterol form the lipid bilayer. A compact mesh of cytoskeleton supports the bi-layer from beneath. α and β-spectrins get arranged head-to head with each other (α-α, β-β) to form individual oligomers and these oligomers lie side-by-side (α-β) in a zipper like fashion to form the heterodimers. Individual heterodimers of spectrin are connected 'head-to-tail' with each other by the actions of the proteins like Actin and Tropomyosin. This network runs beneath the plasma membrane to provide it with the structural stability. The membrane is docked to this underlying spectrin cytoskeleton by the actions of Band 3 and Glycophorin. This docking action is further sealed and strengthened by the actions of the protein 4.1and ankyrin,

1.2.2.2.2 Actin

In the erythrocytes, Actin is organized into short and uniform filaments of approximately 35nm length. On an average six individual tails of the spectrin tetramer complex associate with one of the actin oligomers to form an irregular network with almost a hexagonal lattice (Gilligan and Bennett, 1993). This junction is further strengthened by the association of protein 4.1 and Adducin to form a ternary complex (Gilligan and Bennett, 1993, Conboy, 1993).

1.2.2.2.3 Protein 4.1

About 200,000 copies of Adducin present in each erythrocyte play role in stabilizing the weak spectrin-actin association by interacting, directly with both of the proteins and thus bringing overall stability to the cytoskeleton (Conboy, 1993, Gilligan and Bennett, 1993, Cohen and Foley, 1984).

1.2.2.2.4 Adducin

30,000 molecules of Adducins are present in each erythrocyte and these are chiefly involved in providing structural stability to the membrane cytoskeleton by stabilizing the otherwise weaker interaction between Spectrin and Actin (Anong et al., 2009). Adducin is target of Calmodulin (a calcium dependent protein) and has also been implicated to be involved in forming a novel Band 3-Adducin-Spectrin bridge to connect the Spectrin-Actin-Protein 4.1 junctional complex with the lipid bilayer (Anong et al., 2009, Gilligan and Bennett, 1993).

1.2.2.2.5 Ankyrin

This 1879 amino acid protein, with around 100,000 copies per erythrocyte achieves the mechanical coupling of the membrane skeleton to the lipid bilayer (Mohandas and Chasis, 1993). It simultaneous binds with the specific binding sites at the Band 3 on the bilayer and to the β-spectrin (20nm from the C-terminus) in the cytoskeleton to dock them with each other (Gilligan and Bennett, 1993, Bennett, 1989). The complete interaction is further strengthened by protein 4.2 (Cohen et al., 1993)..

In totality the cytoskeleton proteins form a filamentous network and underlie the lipid bi-layer. This filamentous network is anchored to the lipid bi-layer via the interactions of several integral membrane proteins. This network is responsible for the characteristic membrane elasticity and deformability of the erythrocytes whereas the integral membrane proteins are majorly

responsible for docking it and also work as surface receptors. The absence of the structural protein Tubulin in erythrocyte reasons with the un-involvement of erythrocyte cytoskeleton in cell motility and/or phagocytosis (Mohandas and Chasis, 1993).

The differentiated RBCs do neither phagocytose nor do endocytose and the compact cytoskeleton underlying the lipid bilayer provide it with the structural stability (Mohandas and Gallagher, 2008). It contains only haemoglobin and a few intracellular proteins and lacks biosynthetic activities and/or macromolecular transport. Apparently, it lacks in most of the prerequisites for pathogen survival and development. However for the pathogens those have acquired the ability to survive in this unique environment, RBC can be a 'safe haven'.

RBC lacks in endocytic compartments like lysosomes and hence is incapable of processing and presenting foreign antigen onto the membrane surface and to trigger immune reaction. Thus the intra-erythrocytic pathogens remain largely unrecognized from the immune system.

Nucleated cells are often invaded by both prokaryotic and eukaryotic pathogens. But the fully differentiated human erythrocytes, serve host for a relatively smaller group of pathogens, possibly due to its limited metabolic activities. But for two Apicomplexa parasites of genera *Babesia* and *Plasmodium*, the erythrocytes play obligatory role in development and in the progression of the infection. The choice of mammalian erythrocyte as a host is indicative of the fact that both of these obligatory auxotrophic parasites are highly adapted to this unusual environment, lacking in properties conceivably essential for parasite sustenance.

Additionally as the effects of host cell genetic programme on host-parasite interaction are minimal in case of RBC and its parasites, the parasites and the alterations caused to erythrocyte by them can also broaden our understanding of red blood cell physiology in turn.

1.3 Plasmodium

The apicomplexan protozoa of the genus *Plasmodia are* most well known as the causatives of malaria. Though there are more than 200 species of *Plasmodium* known only five of are known to be causing disease in humans (Ollomo et al., 2009, Perkins and Austin, 2009). Of these, *Plasmodium falciparum* causes the most severe form of the infection, termed malaria tropica, with a higher rate of lethality. *Plasmodium vivax* and *Plasmodium ovale* cause malaria tertiana and *Plasmodium malariae* causes malaria quartana will lesser fatality. The fifth and most recent addition to this list of *Plasmodium*s infecting humans is *Plasmodium knowlesi* (Chin et al.,

1965), reportedly causing an infection with lethal consequences (Cox-Singh et al., 2008, Collins and Barnwell, 2009).

1.3.1 Intra-erythrocytic development of *Plasmodium falciparum*

The life cycle of *Plasmodium falciparum* spans across insect vector, Anopheles mosquito (for sexual stages of development) and human hosts (asexual phases of development).

Once released into the blood stream (during the bite of Anopheles mosquitoes) the sporozoites reach liver and invade hepatocyte. In hepatocytes these undergo exo-erythrocytic development giving rise to merozoites. These merozoites in turn infect erythrocytes, where the parasite completes its asexual development termed as erythrocytic schizogony.

Inside the erythrocyte the parasite remains secluded in the parasitophorous vacuole (PV) delineated by the parasitophorus vacuole membrane (PVM) all along its development. Once inside the erythrocyte the parasite undergoes three morphologically distinct stages of development: the rings (0-24 hours post invasion), trophozoites (24-36 hours post invasion) and schizonts (36-48 hours post invasion).

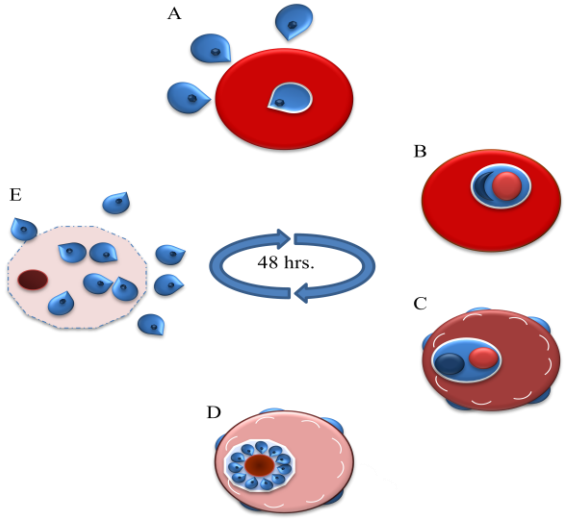


Figure1.3 Intra-erythrocytic development of *Plasmodium falciparum*

A) Erythrocyte recognition and adherence, followed by reorientation of the parasite apical end and invasion. B) The ring stage of the parasite inside erythrocyte C) The trophozoite stage of the parasite inside the erythrocyte, complete with food vacuole and appearance of knob like structures on the erythrocyte membrane D) Schizont stage and clusters of the parasite, severe depletion of the haemoglobin E) Lysis of erythrocyte membrane and parasite egress

Inside the host cell, the merozoites take a flattened, more discoid morphology thus appearing to be 'Rings' under light microscope (0-24 hours post invasion) (Langreth et al., 1978b). These flaunt a collapsed center devoid of any structure with a thicker rim of cytoplasm containing most of the cellular organelle like nucleus, endoplasmic reticulum, mitochondria apicoplast and most of the ribosomes inside (Bannister et al., 2000, Langreth et al., 1978b). From the flattened and discoid ring, the parasites change their shape to a more rounded trophozoite (24-36 hours post invasion) and reach the peak of its metabolic activity. For growth and development, the parasites take up host cell haemoglobin via parasite cytostome and digests it inside the food vacuole, generating a pool of amino acids (Bannister et al., 1975, Bannister and Dluzewski, 1990, Francis et al., 1997).

The indigestible 'haem' fraction is converted into haemozoin crystals and gets accumulated (Francis et al., 1997). The parasites get access to other essential macro and micro nutrients such as glucose, fructose, amino acids (including amino acids missing in the haemoglobin) and purines from the extracellular medium, by altering the permeability of the erythrocyte membrane (Baumeister et al., 2003, Baumeister et al., 2006, Kirk et al., 1994, Staines et al., 2007).

The last phase of the asexual development in *Plasmodium falciparum* is underlined by consecutive and repetitive endo-mitotic nuclear division up-till four times, resulting into a 16 nuclei compound structure named Schizont (36-48 hours post invasion) (Margos et al., 2004). The schizonts dislodge and the erythrocyte is ruptured to release the free merozoites, ready to infect fresh erythrocytes. Some of these merozoites differentiate further into sexually differentiated gametocytes. These gametocytes are subsequently ingested by the female *Anopheles* mosquito during its blood meal and continue on with the sexual phases of the life cycle inside the insect host.

1.3.2 Infection induced alterations of erythrocytes: *Plasmodium falciparum*

Once inside the erythrocyte, the *P. falciparum* modulates the erythrocyte membrane permeability and adhesive properties to facilitate its own survival (Kyes et al., 2001, Deitsch and Wellems, 1996). By protein synthesis and coordinated transport of some of these proteins to erythrocyte cytosol or to the erythrocyte plasma membrane (RBCM) the parasite causes major structural and physiological alterations to the host erythrocyte during its intra-erythrocytic phases of development (Marti et al., 2004, Cooke et al., 2004, Przyborski et al., 2003, Lanzer et al., 2006).

These alterations can range from minor structural changes to major biochemical modifications with significant role in the patho-physiology of the disease itself.

1.3.2.1 Morphological alterations: Knobs and cytoadherence

A prominent alteration in the ultra-structural of the infected erythrocytes is the formation of electron dense 'knob' like structures, beneath the RBCM (Luse and Miller, 1971). These structures are roughly of about 100µm diameter and are reportedly behind the increased cytoadherence of the infected erythrocytes to other healthy erythrocytes and/or infected cells alike (Leech et al., 1984, Udeinya et al., 1981). Moreover the increased adherence leads to resistance against immune-clearance of such cells in spleen (Cooke et al., 2001). As these infected cells adhere to the adjacent vasculatures, they fail to traverse the spleen and therefore are not cleared from the system. The severe clinical outcome and the fatality of malaria, depends much onto this increased adhesion of infected cells to the adjacent vasculatures(Leech et al., 1984).

A protein termed as Knob Associated Histidine Rich Protein 1 (KAHRP1) is essential in the formation of these 'Knob' like structures (Pologe and Ravetch, 1986, Culvenor et al., 1987, Crabb et al., 1997). In studies with the deletion mutants of the *kahrp* gene, the erythrocyte surface was found to be smooth and devoid of any 'knobs' and the cells showed decreased cytoadherence under continuous flow assays (Crabb et al., 1997).

Analysis of the knobs, at a molecular level has further elaborated that the KAHRP self associates underneath the plasma membrane of the infected erythrocytes and interacts with the host cell Spectrin (Kilejian et al., 1991). This forms a platform for *Plasmodium falciparum* Exported Membrane Protein 1 (PfEMP1) to accumulate on and form the backbone of these knobs (Baruch et al., 1995).

The Trypsin sensitive, high molecular mass (200-400kDa) polypeptide of *Pf*EMP1is known to be composed of several extracellular Duffy binding domains (DBL 1-5) interspaced by a) few cystine rich interdomain regions (CIDRs), b) a transmembrane region (TM) and c) an intracellular acidic segment (ATS) (Baruch et al., 1995).Variant forms of the *Pf*EMP1 are known to not only cause the antigenic variation but also help in maintaining specificity towards different host cell receptors (Baruch et al., 2002, Su et al., 1995, Smith et al., 1995). *Pf*EMP3 is believed to be important in trafficking of *Pf*EMP1 onto the surface of the infected erythrocyte, as any

truncation of the EMP3 is reported to be associated with a decreased accumulation of PfEMP1 on RBC surface and decreased cytoadherence in turn (Waterkeyn et al., 2000).

On the cytosolic side of the knobs, several interactions involving the erythrocyte cytoskeleton proteins are believed to provide it with structural stability (Waterkeyn et al., 2000, Glenister et al., 2002, Glenister et al., 2009). Ring-infected Erythrocyte Surface Antigen (RESA) helps in stabilizing this structure by interacting with Spectrin while Mature Parasite Infected Erythrocyte Surface Antigen (MESA) interacts by binding with a junction complex formed by Actin and Band 4.1 (linking the Spectrin dimmers) (Bolte et al., 2009, Maier et al., 2009, Maier et al., 2008). Another suggested role of EMP-3 is binding with Spectrin; an interaction that is non-essential for Knob formation itself but reasoned to be associated with decreasing the deformability of this infected erythrocyte (Waterkeyn et al., 2000, Glenister et al., 2009).

1.3.2.2 Physiological alterations: Novel permeation pathways

Supply of nutrients both in terms of variety and quantity is limited for the parasites of erythrocyte. Moreover the PVM forms a barrier between the parasite and host cytosol, preventing the host cell proteins from accessing the vacuolar space. For some nutrients like few essential amino acids (absent in haemoglobin), fatty acids and pantothenic acid, the parasite largely depends on the extracellular serum. The parasites also need to salvage purine bases from the extracellular media as they lack *de novo* nucleotide synthesis pathways (Gero and O'Sullivan, 1990).

To reach the parasites these nutrients must cross three barriers, from outside-inwards these are a) the erythrocyte plasma membrane (RBCM), b) parasitophorous vacuole membrane (PVM) and c) parasite plasma membrane (PPM). Variety of novel and complex pathways including various transporters, channels and the tubo-vesicular network (TVM) are reportedly involved in the permeation pathways for these nutrients (NPP) (Kirk et al., 1994, Haldar et al., 1994, Upston and Gero, 1995, Lauer et al., 1997, Ginsburg et al., 1983, Kirk, 2001)

Apart from their roles in uptake of essential nutrients, NPP has also been reported to play significant role in the flux of Na^+-K^+ across the infected erythrocyte and in the efflux of solutes (e.g. lactate) from the infected erythrocytes (Poole and Halestrap, 1993, Kirk, 2001, Alkhalil et al., 2004). Digestion of haemoglobin liberates amino acid inside the erythrocyte, creating an

osmotic stress and NPP has been shown to be involved in carrying these amino acids out of the cells thus helping in maintaining homeostasis (Krugliak et al., 2002).

Though the pathways by which these molecules get access to the parasite remains largely unclear but there are three models attempted to explain the phenomenon. The direct access model, proposed by Pouvelle, proposed that the PVM fuses with erythrocyte plasma membrane to allow free access to the molecules and this is intermediated by 'parasitophorous ducts' (Pouvelle and Gysin, 1997, Pouvelle et al., 1991). As an alternative to this model, Ginsburg and colleagues proposed that the increased permeability of the erythrocyte plasma membrane allow the flow of nutrients to and fro (Ginsburg, 1994). The later part of transport was explained by Desai and colleagues as they proposed non-selective pores present on the PVM allow the transfer of these nutrient molecules to the parasite from erythrocyte cytosol (Desai and Rosenberg, 1997). Both of these models of transport into the PV are seemingly incompatible as in the presence of such a direct duct like structure, the erythrocyte stands at a risk of losing out on low molecular weight solutes. The 3rd model proposed by Lauer and colleagues in 1997, suggest that extensions from the PVM reach the proximity of the erythrocyte plasma membrane but doesn't fuse, instead their site of contact acts as a molecular sieve allowing selective uptake of small molecules whereas macromolecules are restricted (Lauer et al., 1997).

1.3.3 Invasion induced compartmentation in erythrocytes

1.3.3.1 Maurer's cleft and Tubovesicular membrane network

Along the development of the parasite inside the erythrocyte, alterations in the erythrocyte cytoplasm become evident. These changes are mostly in terms of the inward and outward trafficking between the parasite, erythrocyte and the extracellular milieu (Aikawa et al., 1986). Attempts by several groups, to understand the altered structures, using confocal microscopy, immunofluorescence and electron-microscopy (EM) had mostly resulted in conflicting outcome.

Maurer's cleft were first described by George Maurer, 1902 but it is only recently after thorough and diverse microscopic analysis, an in depth understanding of the structures had been possible. These are narrated to be slender membranous structures of lamellae containing a lumen and an electron dense coat (Wickert and Krohne, 2007, Langreth et al., 1978a, Atkinson and Aikawa, 1990, Elford et al., 1997) .

Maurer's cleft are presumably involved in sorting of parasite encoded proteins on their final destination to the erythrocyte (Lanzer et al., 2006, Przyborski et al., 2003, Wickert and Krohne, 2007).

Recent data had shown that the Maurer's clefts (MC) are often localized beneath the Knobs and are reportedly anchored to the plasma membrane by some interaction with host cytoskeleton (Waterkeyn et al., 2000, Wickham et al., 2001). This model was further strengthened by the observations that the MC remained attached with the erythrocyte ghosts resulting from osmotic lysis and/or from selective permeabilisation of the erythrocyte membranes (Blisnick et al., 2000, Hanssen et al., 2008).

Proteins of RIFIN (repetitive interspaced family proteins) family (Khattab and Klinkert, 2006), SBP1(Blisnick et al., 2000) MAHRP1 (Membrane associated histidine rich protein) (Spycher et al., 2003), REX1 (ring exported protein) (Hawthorne et al., 2004), REX2 (Spielmann et al., 2006b) and MC-2TMs (Maurer's cleft transmembrane protein) (Sam-Yellowe, 2009) are some of the resident proteins of the Maurer's cleft. Whereas proteins forming a transient association with MC enroute to the erythrocyte plasma membrane are STEVOR (Subtelomeric open reading frame proteins) (Kaviratne et al., 2002, Przyborski et al., 2005), KAHRP (knob associated histidine rich protein) (Wickham et al., 2001), EMP1 (erythrocyte membrane protein) (Waterkeyn et al., 2000)and EMP3 (Knuepfer et al., 2005).

Lipid-fluorescence assay with erythrocytes infected with *Plasmodium falciparum* showed the presence of an extensive network of Tubo-Vesicular Membranes originating from the PVM extending through the cytosol to the erythrocyte membrane (Das et al., 1994, Haldar et al., 1994, Behari and Haldar, 1994, Elmendorf and Haldar, 1993, Elmendorf and Haldar, 1994). In the complete absence of *de novo* biosynthesis of lipids and proteins inside the fully differentiated human erythrocytes, it is highly likely that these membrane enlargements are controlled by some metabolic processes of the parasite itself.

The function of the TVM is not completely understood, however earlier studies had suggested it to be involved in uptake of extracellular lipids (Grellier et al., 1991) and synthesis of sphingomyelin (Elmendorf and Haldar, 1994). Enzymatic inhibition of TVM assembly had been shown to result in the failure of nutrient delivery to the parasite suggesting the role of TVM in nutrient transport as well (Lauer et al., 1997).

Structures like Maurer's clefts and membrane whorls had been distinguished under EM (Langreth et al., 1978a, Aikawa, 1977) whereas lipid-fluorescence assay demonstrated the presence of tubovesicular membranes, extending from the PVM to the erythrocyte plasma membrane (Elmendorf and Haldar, 1994). Tubular extensions projecting from the PVM to the erythrocyte plasma membrane had also been imaged by advanced digitalized fluorescence microscopy and these studies had also suggested the TVN and PVM to be continuous with other intracellular compartments like Maurer's cleft (Grellier et al., 1991) (Elmendorf and Haldar, 1994).

However a contrasting view is that the PVM is distinct from the other intra-erythrocytic membrane bound compartments. Experiments showing the transport of PfEMP-1through the Maurer's cleft suggest that these structures are not connected to the PV (Wickham et al., 2001). Moreover in their experiments with chimerical protein-GFP fusion, Waller and colleagues in 2000 and Wickham and colleagues in 2001 demonstrated that the chimerical-GFP signal was restricted to the vacuolar compartment and could not reach beyond, in turn advocating against the view of a continuum of PVM and TVN (Waller et al., 2000, Wickham et al., 2001).

1.3.3.2 The parasitophorous vacuole: an unusual compartment

To maintain their cellular entities, intracellular parasites live secluded (at least temporarily) inside unique vacuolar compartments. Depending upon the nature of host cell invasion these compartments attain unique features. In phagocytic cells, these compartments are often derived from the phago-lysosomal membrane system of the host cell; as seen in *Leishmania* spp where the parasite resides and replicates within such a vacuole while drawing nutrients from the incoming traffic of host cell endomembrane system (Haas, 2009).

Contrary to this, during the active invasion of the host cells, obligate intracellular apicomplexan parasites of genera *Plasmodium* and *Babesia* form a unique vacuolar compartment. Erythrocytes are devoid of any endo/phagocytic activity; hence this compartment is most certainly deprived of any component of the endo/phagosomal membrane system. To be termed distinctly from any endo-phagocytic vacuole, this is called as parasitophorous vacuole (Scholtyseck and Piekarski, 1965) with the surrounding membrane called parasitophorous vacuolar membrane (PVM) The PV provides the parasites with the desired seclusion from host cell cytoplasm and maintains a neutral pH (Lingelbach and Joiner, 1998). All along the intra-erythrocytic growth of the

Plasmodium spp., the PVM forms a pathogen-host cell interface and the vacuolar space of PV represents a unique proteome; different from both the erythrocyte cytosol and the parasite cytosol (Nyalwidhe and Lingelbach, 2006). Such a safe niche confers resistance against some of the host cell defences but could also cut the parasite (at least temporarily) off from host metabolites; the parasite circumvents this by remodelling the vacuole, making it permissive to vital substances (Lauer et al., 1997, Desai and Rosenberg, 1997).

1.3.3.2.1 Formation of the parasitophorus vacuole

The PV is formed upon the invasion of red blood cells by the merozoites of *Plasmodium falciparum*. These zoites are characterised by the presence of some unique sub-cellular structures like a) an Inner Membrane Complex (IMC) located just beneath their parasite plasma membrane and constituted of joined and flattened cistarnae (Porchet-Hennere and Nicolas, 1983), b) an Apical Complex called Conoid, sustaining the micro-tubular cytoskeleton of the parasite (Morrissette and Sibley, 2002), c) a non-photosynthetic plastid, presumably acquired by secondary endosymbiosis from some ancestral alga and involved in synthesis of neutral lipids and isoprenoids (Marechal and Cesbron-Delauw, 2001) and d) a set of 3 secretory organelle named micronemes, rhoptries and dense granules (Dluzewski et al., 1992, Ward et al., 1993). The formation of the PVM is concomitant with the release of components from these specialized secretory organelles; hence these organelles are believed to be the key players in the formation of the PVM [review by (Saffer et al., 1992, Schwartzman and Saffer, 1992)].

The erythrocyte invasion by the parasite is a rather quick process lasting on an average of 30-60 seconds (Cowman and Crabb, 2006) and in next 10-15 minutes the invading parasite alters their shapes forming further intercellular stages (Bannister and Mitchell, 2003).

The initial recognition of the non-infected erythrocytes and subsequent random attachment of the merozoite to it is a reversible interaction mediated by a number or proteins, of both erythrocyte and merozoite origin (Bannister and Dluzewski, 1990). Once the initial attachment is secured, the merozoite re-orients its apical end (containing rhoptries, micronemes and dense granules), comes in contact with the erythrocyte surface and forms a stable, compact yet movable association known as the ‘tight junction’(Aikawa et al., 1978). Stimulated by some yet undefined stimuli, the contents of the microneme are released followed by the contents of the dense

granules thus initiating a successful invasion (Aikawa et al., 1978, Aikawa et al., 1981, Bannister and Mitchell, 2003, Bannister and Dluzewski, 1990)

Plasmodium falciparum Apical membrane antigen-1 (PfAMA1) is believed to be a key link between the initial weak surface contact and the compact tight junction that propels the host cell invasion (Cowman et al., 2002, Alexander et al., 2006, Triglia et al., 2000). Parallel to this, the Duffy Binding like (DBL) protein family and *Plasmodium falciparum* Reticulocytes Binding Protein Homolog (PfRh) act as adhesins in this junction (Miller et al., 2002a, Miller et al., 2002b, Camus and Hadley, 1985). Ultrastructure analysis has revealed that during the movement of the invading merozoite, a membrane lined invasion pit, the parasitophorous vacuole, is created from the borders of the junctional bands and the contents of the rhoptries are discharged into it (Bannister and Mitchell, 2003, Sam-Yellowe et al., 1995, Aikawa et al., 1978, Aikawa et al., 1981).

Completion of invasion is underlined by a sequence of events starting with the sealing of the PV by fusion of the RBC membranes, lining across the mouth of the pit and subsequent detachment of the PV from the erythrocyte surface, followed by the movement of the dense granules (DG) to the merozoite surface and their fusion with the membrane and releasing of their contents into the PV, leading to further expansion of it (Aikawa et al., 1978, Bannister and Dluzewski, 1990).

The complete biochemical composition and the molecular details underlining the formation of PV or PVM however are still not clear. Presumably, erythrocyte membrane components are modified at the point of invasion by a process where the secretions of parasite apical organelle play an important role.

1.3.3.2.2 Components of the plasmodial PVM

The PVM is a large membrane with an estimated surface area of 30-33μm^2 (Suss-Toby et al., 1996) and is formed *de novo* within 10-20 seconds; hence its formation implies the involvement of some unusual biological process. The PVM reportedly originates from the erythrocyte plasma membrane but the constituents are altered; presumably by the lipid materials secreted from the parasite rhoptries (Bannister and Mitchell, 2003). Moreover as the parasite grows and matures within the erythrocyte inside the PV, the PVM also needs to expand to a considerable extent, in order to accommodate the parasite. Absence of any lipid bio-synthesis, on part of the erythrocyte, it is most likely that either the endogenous phospholipid biosynthesis or the lipid

scavenging of the invading parasite helps in maintaining the supply of lipid precursors and to maintain the PVM in the infected erythrocytes (Spielmann et al., 2012, Gruring and Spielmann, 2012).

1.3.3.2.2.1 Lipids of the PVM

The role of the parasite rhoptry, either as the storehouse of membrane lipids helping in the *de novo* membrane biogenesis or as the supplier of internal membrane is under much debate, however the quick and successive events during the invasion of *Plasmodium falciparum* leading ultimately to the formation of the PVM, most certainly demonstrate some unique cell biological phenomenon with little precedence.

The lipid constituents of the PVM are not well known and neither are the lipid components of *Plasmodium* rhoptries had been well characterised. In 1981 Aikawa and colleagues, demonstrated the involvement of rhoptry components in the formation of the PV with Cytochalasin treated *Plasmodium* spp. zoites. Cytochalasin blocks parasite entry but does not affect rhoptry discharge. After the invasion assay with Cytochalasin treated zoites, relatively smaller but empty vesicular structures were reported inside the parasite cytoplasm, even though the parasite entry was blocked as a result of the treatment (Aikawa et al., 1981).

Recent studies using non-exchangeable lipid analog have shown that the PVM lipids are largely derived from the erythrocyte membrane. These experiments used non-infected erythrocyte and labelled those with non-exchangeable fluorescent lipid probes, thereafter allowed parasite invasion. By showing the presence of labelled lipid components on the PVM these experiments confirmed that the PVM lipid is largely derived from the erythrocyte (Ward et al., 1993, Pouvelle et al., 1994). However Dluzewski and his colleagues in 1995 contradicted these findings by showing no apparent loss of the surface area in the newly infected erythrocytes (Dluzewski et al., 1995).

A more ‘unifying hypothesis’ could be that the rhoptry lipids indeed take part in the formation of the PVM but erythrocyte plasma membrane does also supply a major portion of lipids to help forming the PVM [reviewed in (Lingelbach and Joiner, 1998)]. Extensive remodelling of the erythrocyte phospholipids components was proposed following the experiments of Murphy and colleagues (2007), as they showed that the major phosphoinositide of RBC membrane, Phosphatidylinositol- 4 5-bisphosphate was excluded from the PV (Murphy et al., 2007).

The fact that some of the host cell cholesterol rich membrane microdomains (arguably the preferred entry sites for the parasite) associated proteins and excluded from the newly formed PVM together with the finding of the presence of some of the parasite rhoptry associated proteins into the newly formed PVM clearly indicates a mixed origin of the lipids present in the PVM as well (Hiller et al., 2003, Murphy et al., 2004) and strengthens the unifying hypothesis.

1.3.3.2.2.2 Proteins of the PVM

A number of proteins found present on the PVM and further onto the TVM of *P. Falciparum*-infected erythrocytes have now been identified to be of parasite origin and reportedly these proteins had been transported to these final destinations directly from the parasites secretory pathways (Maier et al., 2009). These proteins include, one of the earliest known of the PVM proteins, a type I single pass transmembrane protein Exported Protein-1 (EXP-1) (Hope et al., 1984, Gunther et al., 1991). Initially EXP-1 had been identified by a monoclonal antibody, as an antigen reactive to both sporozoites and blood stage of the parasites (Hope et al., 1984) and later the PVM localization was confirmed by immuno-electron microscopy by different groups (Simmons et al., 1987, Kara et al., 1989) .EXP-1 carries a cDNA sequence similarity to the major tetramer repeat of the circumsporozoite protein, hence is also known as circumsporozoite protein-related antigen (Hope et al., 1984, Coppel et al., 1985).

The organization of the EXP-1 on the PVM with its N-terminal facing the vacuolar side and the C-terminal facing the cytosol (Ansorge et al., 1997) is consistent with the hypothesis about the delivery of such proteins by vesicular budding, originating at the parasite plasma-membrane and subsequently fusing with the PVM [reviewed by (Lingelbach and Joiner, 1998)]. *Pf*EXP-1 presumably plays a key role in the asexual blood stages of the parasite (Maier et al., 2008, Spielmann et al., 2012).

Another group of PVM resident proteins, presumably been directly targeted to the PVM are the Early Transcribed Membrane Proteins (ETRAMPs) (Spielmann and Beck, 2000, Spielmann et al., 2003). 14 proteins from this family are known to be present in the *Plasmodium* making the ETRAMPs a most prominent of the proteins in blood stage of PVM (Spielmann et al., 2003, Spielmann et al., 2012, Spielmann et al., 2006a). Proteins of this group of are highly charged and display some structural similarity to that of EXP1 (Spielmann et al., 2003). The C-terminus of ETRAMPs face the host cell cytosolic side as well; however they form membrane dependant

homo-oligomeric complexes, distinct from the EXP-1 oligomers (Spielmann et al., 2003, Spielmann et al., 2006a)

Different ETRAMPs had been reported to show stage specific expression in the blood stages of the *Plasmodium falciparum,* indicating their customized role for the intra-erythrocytic stages of this parasite (Spielmann et al., 2003, MacKellar et al., 2011). There is no clear evidence of any larger distribution of the *Pf*ETRAMPs inside the erythrocyte, beyond the PVM but *Pf*ETRAMP10.3 had been found present also in structures resembling Maurer's clefts in trophozoites and schizonts (Mackellar, O'Neill et al. 2010).

The specific roles of ETRAMPS, in the blood stages of the parasite is yet not clear but its abundance and capacity of forming independent membrane domains, suggest a possible involvement in structural rearrangement and membrane partitioning had been suggested. (Mackellar et al., 2010) A parallel involvement of the ETRAMPS had been suggested, in and as organizers of specific membrane domains and export of the domains into the host cell (Spielmann et al., 2003, Spielmann et al., 2012, Mackellar et al., 2010). It is also likely to be involved in generation of smaller vesicular structures and TVM (Spielmann et al., 2012).

A high molecular mass *Plasmodium falciparum* rhoptry associated protein RhopH complex, (consisting 3 domains, RhopH1, 2 and 3) is reportedly transferred from the apical organelle and is found present on the PVM of the newly infected ring stage of the parasites (Lustigman et al., 1988). RhopH2 is known to be delivered directly to the PVM (Hiller et al., 2003) whereas the RhopH1, which is responsible for causing the major variations in the RhopH complex, had been detected in the periphery of the ring stages of the parasite (Kaneko et al., 2005, Ling et al., 2004) The RhopH1 is represented by the proteins coded by one of the many members of the Cytoadherence Linked Asexual Gene family (CLAG) (Ling et al., 2004).

Independent studies have also reported the presence of RhopH components and CLAG proteins in different host cell compartments (Vincensini et al., 2008, Nguitragool et al., 2011). Though the RhopH complex is reportedly lost during the ring stages of development, causing doubt about its specific role in the PVM; the CLAG-3 has been reported to be associated with and involved in the NPP of *Plasmodium falciparum* infected cells (Nguitragool et al., 2011).

A major function of the PVM in the erythrocytic phases of the parasite is mediating the export of the proteins into the host cell. Using proteome analysis of the parasite DRMs and prediction

tools, De Konig-Ward and colleagues in 2009 identified a strong candidate for this protein translocation machinery, named *Plasmodium falciparum* trasnlocon for exported proteins (PTEX) (de Koning-Ward et al., 2009). The PTEX proteins are supposedly transferred from the dense granules to the newly forming PVM during the merozoite invasion (de Koning-Ward et al., 2009, Bullen et al., 2012)

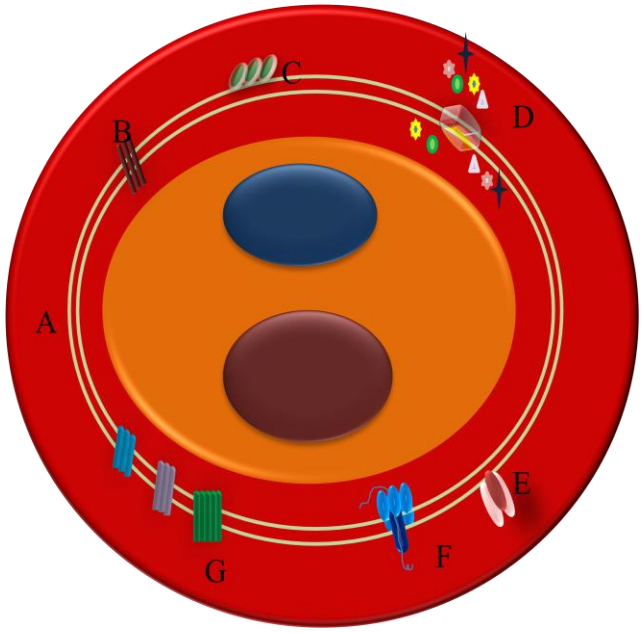


Figure 1.4 Model for the *Plasmodium falciparum* proteins found present in the PVM during the asexual blood stages Adapted from, Molecular make-up of the *Plasmodium* parasitophorous vacuolar membrane (Spielmann et al., 2012)

A) The PVM B) EXP-1 oligomers C) Stomatin Oligomers D) Solute pores E) RhopH/CLAG, F) PTEX-Exported protein G) Different ETRAMP oligomers.

PTEX is reportedly constituted of 5 interacting partners:(de Koning-Ward et al., 2009) a) Exported protein 2 (EXP 2),(Johnson et al., 1994) b) the ATP binding subunit HSP101 (de Koning-Ward et al., 2009) c) the redox protein Thioredoxin 2 (TRX 2) and two hypothetical proteins d) PTEX88 and e) PTEX150 (de Koning-Ward et al., 2009, Spielmann et al., 2012). The EXP2 is proposed to be forming the actual pore whereas the HSP101 is involved in the unfolding activity (de Koning-Ward et al., 2009, Bullen et al., 2012)

Co-immunoprecipitation experiments had shown an association of the PTEX with proteins carrying typical *Plasmodium* Export Element (PEXEL) suggesting their active involvement in the protein translocation (de Koning-Ward et al., 2009).

Several other apical complex associated proteins reportedly are also transferred to the ring stage upon invasion, but their localization has not always been ascertained. This include postulated components like a) low molecular weight Rhoptry complex (RAP1-3) (Howard et al., 1998, Baldi et al., 2000) b) Rhoptry associated membrane antigen (RAMA) (Topolska et al., 2004, Richard et al., 2009) and confirmatively PVM associated components c) *Plasmodium falciparum* Stomatin homolog (Hiller et al., 2003).

In a contrast to the erythrocyte membrane lipids, erythrocyte membrane proteins were initially believed to be completely excluded from the PVM (Dluzewski et al., 1992, Ward et al., 1993). This hypothesis however had been contested in recent past and now it been established that several of the erythrocyte membrane proteins are internalized during the invasion and are present on the PVM. In the recent past, resurgence of the concept or biochemically and functionally distinct microdomains within the plasma membrane of cells (Brown and London, 1998a, Brown and London, 1998b), including those off erythrocytes (Lingwood and Simons, 2010, Yu et al., 1973) have led to revaluation of the protein contents of the PVM. As representatives of these unique microdomains, analysis on detergent resistant microdomains obtained from the total membrane fractions of the infected erythrocytes, has revealed several proteins to be associated with the PVM (Murphy et al., 2004). The erythrocyte membrane proteins found associated with the PVM, include GPI anchored-micro domain associated proteins like Flotillin 1 and 2, CD 59 and multiple spanning membrane proteins Aquaporin 1 and 3 alike (Lauer et al., 2000, Murphy et al., 2007, Murphy et al., 2006, Bietz et al., 2009). Whether such proteins are present on the PVM due to their incomplete exclusion or these are actually taken up in during the invasion to perform specific roles needs further confirmation (Spielmann et al., 2012). Inclusion of some of the parasite rhoptry derived proteins onto the newly formed PVM and exclusion of some of the erythrocyte membrane proteins known to be associated with the cholesterol rich microdomains (presumably preferred entry sites of the parasites) from the PVM implicates the participation of both host and parasite derived proteins in the formation of the PV (Hiller et al., 2003).

1.3.3.2.3 Functions of the PV

1.3.3.2.3.1 Transitional compartmentation

In absence of any cellular organelle or indigenous protein trafficking machinery inside the erythrocyte, the parasites must actively regulate and mediate the protein transport within the

erythrocyte to regulate the uptake of nutrients from the extracellular surroundings and also to display parasite coded proteins onto the erythrocyte surface to modulate infection (Charpian and Przyborski, 2008, Cooke et al., 2004).

The protein trafficking to the PV is similar to that of the classical pathway of the higher eukaryotes, starting with the proteins entering the ER based on their N-terminal targeting signal sequence (Wickham et al., 2001, Johnson et al., 1994). Some of the proteins, like the proteins of Serine-Rich Antigen (SERA) family remain inside the vacuolar lumen (Delplace et al., 1988, Knapp et al., 1989) whereas some others such as KAHRP are directed further outwards towards the erythrocyte plasma membrane (Wickham et al., 2001).

Soluble parasite proteins destined for different locations in the host erythrocyte reportedly to pass through the PV lumen before being secreted into the cytoplasm (Johnson et al., 1994, Ansorge et al., 1996). It is now well established that these exported proteins possess some conserved motifs (RxLxE/Q/D), otherwise known as *Plasmodium* export element (PEXEL) or Host Cell Targeting (HCT)/Vacuolar Targeting Sequence(VTS) helping in the targeting beyond the PVM into the host cell (Hiller et al., 2004, Marti et al., 2004). This PEXEL sequence gets cleaved inside the parasite ER and this modified N-terminus gets acetylated (Boddey et al., 2009, Chang et al., 2008).

The machinery or the mechanism for this transport is not completely clear. Within the PV the PEXEL-containing chimeras had been shown to form bead like appearance and were found resistant to photo bleaching, which led to the suggestions about the presence of some sub-compartments within the vacuole allegedly housing some of the components of this translocation machinery (Wickham et al., 2001, Adisa et al., 2003). In 1996 Ansorge and colleagues showed that the PVM harbours an ATP-dependant translocation activity (Ansorge et al., 1996). This involvement was further strengthened by the findings of Gehde and colleagues in 2009 as they showed that the exported proteins need to get unfolded in order to get transported through the PVM (Gehde et al., 2009) and in the same year de Konig-Ward and colleagues, using proteome analysis of detergent resistant parasite membrane fractions and prediction tools identified the strong candidate for this protein translocation machinery termed *Plasmodium falciparum* Trasnlocon for Exported Proteins (PTEX) (de Koning-Ward et al., 2009).

1.3.3.2.3.2 Nutrition acquisition and maintenance of the iconic environment

By the schizont stage almost 80% of the host cell haemoglobin is digested by *Plasmodium falciparum* in order to obtain the necessary amino acids and also to make 'space' for the growing parasites (Lew et al., 2003). The parasite cytostome actively phagocytose the PVM with cytosol to obtain cytoplasmic haemoglobin and transports it into the food vacuole for digestion (Elliott et al., 2008). Though the major nutritional requirement are met from haemoglobin but for the essential amino acids like isoleucine (absent in haemoglobin) or cystine, methionine and glutamine (all three are scarcely available in haemoglobin) and also for other essential nutrients (purines) the parasites need access to the external environment.

Non-selective pores allowing passive bi-directional movement of small molecules (up to 2000 Da) had long been proposed by different research groups (Desai and Rosenberg, 1997, Kirk, 2001, Nyalwidhe et al., 2002). But for the components with higher molecular weight, the mechanism for transport is more likely to be the NPP (Saliba and Kirk, 2001, Kirk and Saliba, 2007).

To perform the functions of 'Molecular sieve', the ionic composition of the PV is expected to be similar to that of the erythrocyte cytosol, but such an iconic strength in the vacuolar space could be well detrimental for the parasite. The RBC cytoplasm has a high concentration of K^+ (140mM) but, in a clear contrast to the regular extracellular medium, it has a very low concentration of Ca^{++} (100nM) (Alleva and Kirk, 2001). Eukaryotic cells need an extracellular Ca^{++} concentration close to the millimolar level and this is definitely unavailable to the *Plasmodium falciparum* inside the erythrocyte. The parasite achieves the necessary osmolarity by maintaining a high Ca^{++} (40µM) concentration within the PV (Gazarini et al., 2003).

A concentration like this is easily compatible with the native intracellular Ca^{++} concentration required a healthy *Plasmodium falciparum* and at the same time is 100-1000 fold higher than the Ca^{++} concentration found inside the parasite cytoplasm and the erythrocyte cytoplasm respectively (Gazarini et al., 2003). It was also demonstrated that a reduction in the Ca^{++} concentration in the PV, initially impairs the development and later becomes detrimental for the parasite survival (Gazarini et al., 2003).

Analysis of *Plasmodium falciparum* genome has resulted in identification of many a signalling protein, similar to the ones seen in vertebrates and believed to be involved in Ca^{++} signalling in

the same (Garcia et al., 2008). With many other factors equally involved in maintaining the Ca^{++} the *Plasmodium falciparum* makes the PV by itself act as an intra-erythrocytic but extra-cellular (parasite) Ca^{++} reservoir for its sustenance.

1.3.3.2.3.3 The PV and merozoite egress

To continue on with the infection the parasite egress the host cell and infect healthy cells. The invasion lead to formation of the PV and this egress leads to the destruction of it and also of the erythrocyte membrane. Parasite egress is believed to be a rapid and highly regulated process [Review in (Dowse et al., 2008)]. Inhibitor assays and live cell imaging has revealed the fact the merozoite egress of *Plasmodium falciparum* is a two step process but whether it's the PVM or the erythrocyte membrane that ruptures first is still unclear (Wickham et al., 2003, Glushakova et al., 2005). Studies on liver stage merozoite egress, as a parallel to the blood stages, have strengthened the idea of PVM breakdown preceding erythrocyte membrane rupture (Wickham et al., 2003, Heussler et al., 2010, Sturm et al., 2006).

There are many models explaining the mechanism and explaining the molecular mechanism of egress and all these all independently highlight involvement of proteases. Treatment of the *Plasmodium falciparum* asexual blood stage cultures with various protease inhibitors like Leupeptin, Chymostatin, Antipain (Serine and cystine protease inhibitor), E64 (a high specificity cystine protease inhibitor) Pepstatin (Aspartic protease inhibitor) have all resulted in blocking of the merozoite egress, leading to the idea that the egress is a thoroughly protease dependant process [Review in(Blackman, 2008)]. Though the details of the processes are not yet clear, it is certain that the breakdown of the PVM and the erythrocyte membrane are differentially regulated.

SERA proteins, the members of a nine gene family, are found to be most highly expressed at the schizont stages of the parasite and are found localized on the PV lumen, making them one of the potential mediators of the egress of *Plasmodium falciparum* (Delplace et al., 1988, Knapp et al., 1989, Miller et al., 2002b). This family of proteins reportedly contain a rather conserved central papaine-like domain and some conserved cystine residues in their N- and C-terminals (Miller et al., 2002b).

The SERA5 is processed by a subtilisin-like serine protease named PfSUB1 (Blackman, 2008, Blackman et al., 1998, Sajid et al., 2000, Yeoh et al., 2007); a processing that had previously

been implicated to be associated with the egress of the blood stage parasite (Delplace et al., 1988). Using transgenic parasite lines, the expression of *Pf*SUB1was monitored and the *Pf*SUB1 was demonstrated to be released into the PV space from dense granule like organelles (exonemes), just prior to the egress (Yeoh et al., 2007). Upon its release into the PV, the *Pf*SUB1 reportedly mediates the proteolytic processing of three major merozoite surface antigens, MSP1, MSP6 and MSP7 (Koussis et al., 2009). Selective inhibition of *Pf*SUB1 was found to be preventing the egress and also blocking the processing of SERA5 (Koussis et al., 2009).

A more recent finding is the parasite mediated hijack of host cell calcium regulated Calpain protease in order to facilitate the parasite escape (Chandramohanadas et al., 2009). In the respective experiments it was shown to be achieved by a controlled mechanism involving Ca^{++} based signalling (Chandramohanadas et al., 2009). However the parasite mediated modulation of the host cell Ca^{++} signalling itself leaves much room for research.

Thus the PV acts as a host of factor(s) regulating the parasite egress and/or mediating proteolytic remodelling of the merozoite, preparing it further for its egress. As a unique and almost unparallel biological compartment and its surrounding membrane, the PV and its surrounding membrane the PVM most definitely have unique features that need thorough and multidimensional research for a greater understanding.

1.4 Babesia

Babesia belongs to the suborder *Piroplasmidea* and family *Babesiiadae* and this classification had been based on the traits like: a) invasion of erythrocytes only, b) multiplication within the erythrocyte in a process that is more similar to budding and binary fission than schizogony and c) the lack of haemozoin as a digestive waste. Babesiosis is the infection, caused by the Apicompexan parasites of genus *Babesia* and is most common amongst the infectious diseases in the free-living animals (Telford and Spielman, 1993, Yokoyama et al., 2006). *Babesia* can also cause opportunistic infection in immuno-compromised humans (Homer et al., 2000, Gorenflot et al., 1998). Since the first human fatality caused by *Babesia divergens* was reported in 1956, it has increasingly gained interest owing to the zoonotic nature (Quick et al., 1993, Skrabalo and Deanovic, 1957, Montero et al., 2006, Spielman et al., 1985, Gorenflot et al., 1998).

Although several species of *Babesia* had reportedly been found involved in the infections of humans but the rodent parasite *Babesia microti* and the bovine parasite, *Babesia divergens* have

been found responsible for most of the human infections reported this far (Spielman et al., 1985, Gorenflot et al., 1998).

Like most other Apicomplexa, the life cycle of *Babesia* as well involve two hosts. In the invertebrate vector, mostly ticks (almost all Ixodids), *Babesia* completes the sexual phase of development whereas in the mammalian host it completes the asexual phases of development. Inside the tick host the parasite can continue either with a transovarial transmission, creating infectious progeny or with a transstadial cycle, where the individual ticks retain the infectivity through different stages of the lifecycle (Hunfeld et al., 2008).The major differences amongst *Babesia* itself is their choice and capability of this transovarial transmission (Hunfeld et al., 2008)

1.4.1 Intra-erythrocytic development of *Babesia*

The sporozoites of *Babesia* enter a healthy mammalian host during the blood meal of the tick and can directly infect the erythrocytes and undergo asexual phases of development in these (Hoyte, 1965, Hoyte, 1971, Mosqueda et al., 2002). After the initial contact and reorientation, *Babesia* penetrates into the erythrocyte and form a parasitophorous vacuole, in which it remains transiently encapsulated (Rudzinska et al., 1983, Rudzinska et al., 1976).

Unlike in *Plasmodium*, the PV in *Babesia* is a temporary compartment and dissolves soon after parasite invasion in a yet unknown molecular mechanism (Rudzinska, 1976, Hines et al., 1995, Hines et al., 1992). Components of the 'Spherical body', a *Babesia bovis* compartment similar to the typical 'Dense granules' of other Apicomplexan are believed to be involved in the lysis of the PV and bringing the parasite in direct contact with the erythrocyte cytosol (Hines et al., 1995).

Inside the erythrocyte the parasite grows by binary fission (Lobo et al., 2013, Lobo et al., 2012). The fissions give rise to two to four separate merozoites also referred as paired forms and tetrads/Maltese cross respectively (Cursino-Santos et al., 2014a, Homer et al., 2000). The multiplication inside the erythrocyte is by single or multiple rounds of binary fissions, leading ultimately to merogony.

During their stay inside the erythrocyte, the parasites feed on the haemoglobin and also on the nutrients drawn in from the serum, to meet their metabolic needs (Rudzinska, 1976). These metabolic activities cause physiological and biochemical alterations of the infected cell and can

range from facilitating the nutrient acquisition of the parasite to helping in evading cytoadhesion and immune-clearance (Lobo et al., 2013).

These daughter cells egress from the erythrocyte by a 'perforin-like protein' dependent process and continue on with further infection (Kafsack and Carruthers, 2010). Besides forming further merozoites, a fraction of invading merozoites mature and differentiate inside the erythrocyte to form gametocytes. These gametocytes float around in the blood and are taken up by the tick during its next blood meal to continue on with the sexual phases of the life cycle.

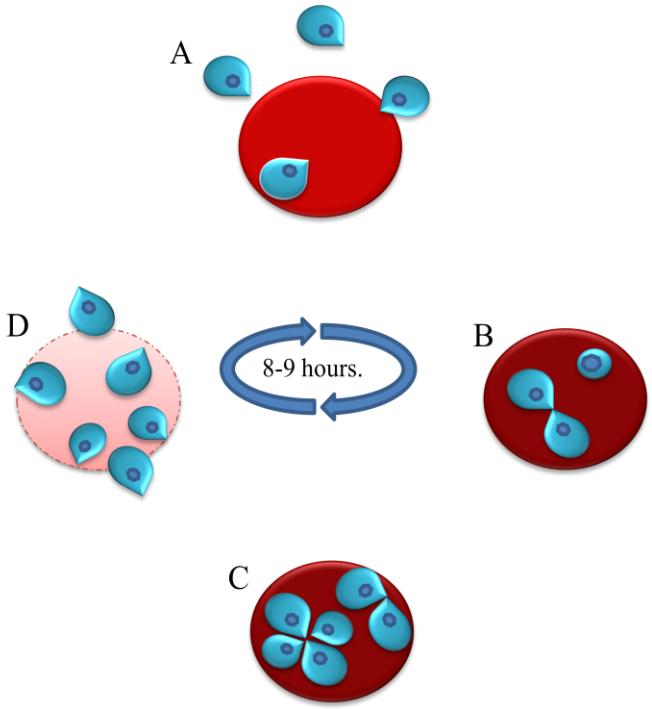


Figure1.5 Intra-erythrocytic development of *Babesia divergens*

A) Erythrocyte recognition and adherence, followed by reorientation of the parasite apical end and invasion B) Multiple infection with one invading parasite already in the piroplasm form and other in ring stage C) The budding like development of *Babesia divergens* resulting into two or four individual parasites, typically arranged as dumbbells or as Maltese Cross D) The piroplasms dismantle the clusters and rupture the membrane to egress.

1.4.2 Infection induced alterations of erythrocyte: *Babesia*

In general principle, infected host cells show properties not typically found in non-infected cell. Haemosporidian *Babesia divergens* infects the differentiated erythrocyte, much like the related *Plasmodium falciparum*. The stark resemblance of the pathology and clinical outcome of malaria (caused by *Plasmodium falciparum*) to the cases of severe babesiosis in cattle, (caused by *Babesia bovis* and *Babesia divergens*) led to their inevitable comparison (Hutchings et al., 2007,

Allred, 1995, Clark and Jacobson, 1998, Cooke et al., 2005). Some of these alterations, caused to the infected erythrocyte by *Babesia* spp. were found to be similar in line to those found in *Plasmodium* spp.infected ones.

1.4.2.1 Morphological alterations: Ridges and cytoadherence

Reportedly, during the infections of *Plasmodium falciparum* and *Babesia bovis*, the infected erythrocytes were often found extensively sequestered in the microvasculature, leading ultimately to cerebral babesiosis or cerebral malaria and/or multi-organ failure causing subsequent fatality (Parrodi et al., 1989, Everitt et al., 1986, Cooke et al., 2001). When studied in the less fatal infections of *Babesia bigemina* or *Plasmodium vivax*, neither the intra-vascular accumulation nor the cerebral vaso-occlusive complications were found (Hutchings et al., 2007). In order to understand the altered properties of the erythrocytes infected by *Babesia bovis*, extensive *in vitro* analysis was conducted by Parrodi and his colleagues in 1989 and Hutchings and his colleagues in 2007. Their findings have demonstrated an increase in the adhesion properties of these infected erythrocytes; a finding that much in line with similar findings by Cooke et al in 2001 in *Plasmodium falciparum* infected erythrocytes (Parrodi et al., 1989, Wright et al., 1989, Cooke et al., 2001, Hutchings et al., 2007). Whereas a similar study by O'Connor in 1999 with erythrocytes infected with *Babesia bigemina* (known to much less of a fatal infection) demonstrated the non-adherent nature of such erythrocytes (O'Connor et al., 1999)

Electron dense structures duly named as 'Knobs' had been well described to be associated with cytoadherence of cells infected with *Plasmodium falciparum* (Luse and Miller, 1971). Transmission EM and freeze-fracture EM performed on *Babesia bovis*. infected cells demonstrated the presence of similar but more 'elliptical' or 'satellite' like protrusions on the surface of the erythrocytes infected with *Babesia bovis*; but these structures were relatively bigger (320nm*160nm) than the typical 'knobs' (150nm*56nm) of *Plasmodium falciparum* infected cells and were described to be as the contact points of the parasite to the endothelial cells both *in vivo* and *in vitro* (Hutchings et al., 2007, O'Connor et al., 1999, Cortes et al., 2005, Cooke et al., 2005)

Using Atomic force microscopy (AFM) Hutchings and his colleagues (2007) compared the erythrocytes infected with *Plasmodium falciparum* to the ones infected with *Babesia bovis*, and confirmed that the previously described satellite like protrusions on cells infected with *Babesia*

bovis, were rather 'elongated' and were more 'ridge' like in morphology (Hutchings et al., 2007). They also proposed a correlation between the numbers of such ridges to the virulence of the parasite (Hutchings et al., 2007).

As a supportive to this model they demonstrated complete absence of such ridges on erythrocytes infected with *Babesia bigemina*, where lack of in vivo cytoadherence or sequestering had already been confirmed (O'Connor and Allred, 2000, Hutchings et al., 2007)

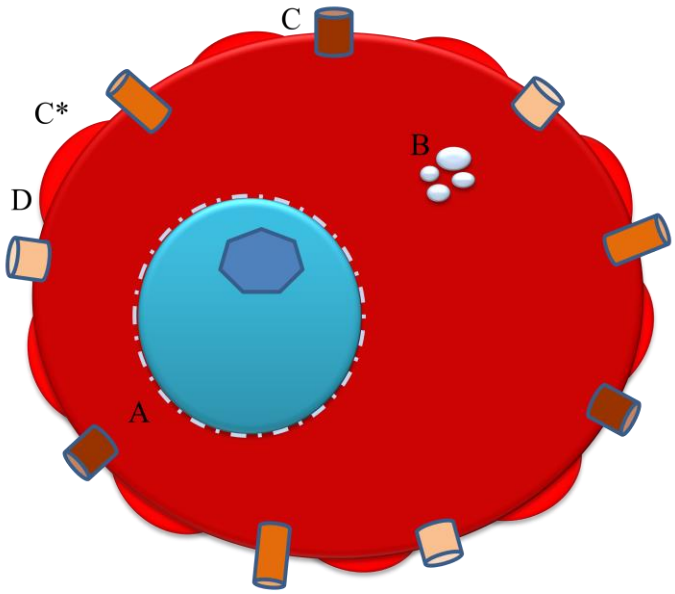


Figure 1.6: Infection induced modifications in erythrocytes infected with *Babesia divergens*

A) The PV, dismantled B) Vesicular compartments of *Babesia* infected erythrocyte C) Parasite encoded proteins mediating NPP, exported to the erythrocyte membrane, C*) Erythrocyte endogenous transporter proteins mediating NPP, activated post invasion D) Ridges

In erythrocytes infected with *Plasmodium falciparum*, the increase in cytoadherence and subsequent sequestering had been shown to have resulted from the recognition of various proteins like CD36, ICAM-1 and Chondroitin sulphate-A; expressed on the vascular endothelial cells (Cooke and Coppel, 1995, Cooke et al., 2001, Cooke et al., 2005). However in erythrocytes invaded by *Babesia* spp. no such increased affinity towards the cell surface CD36 was reported (Hutchings et al., 2007).

In *Plasmodium falciparum*, a parasite Knob Associated Histidine Rich Protein (KAHRP) had been described to be self aggregating beneath the erythrocyte surface and forming the typical knob like protrusions (Crabb et al., 1997, Chishti et al., 1992). However no homolog of the

Plasmodium falciparum KAHRP had been reported in *Babesia* spp. so far, and unlike the knobs of *Plasmodium falciparum* infected cells, the ridges on cells infected with *Babesia bovis* were found to be collapsing on treatment with trypsin, suggesting that the ridge forming proteins can be exposed on the erythrocyte surface to a varied extent (Hutchings et al., 2007). However, under flow assay experiments these trypsin treated cells were shown to possess enhanced adherence to the cells of endothelium and to platelets, indicating the presence of some autonomous membrane rearrangement in these cells that exposes tripsin tolerant patches or zone to evade immune clearance (Hutchings et al., 2007). These facts put together, indicate towards some unique mechanism underlying the formation of ridges in *Babesia*, those are yet to be completely understood.

Based on the results (of different groups) of various immunoprecipitation assay with *Babesia bovis*-iRBC, it is known that the *Babesia bovis* Variant Surface Antigen 1 (VESA1), known to accumulate over abnormal surface protrusions, play an active role in increasing the adherence of the infected cells to endothelial cells (Al-Khedery and Allred, 2006, Hutchings et al., 2007). Transcribed from the multigene family of *ves*, similar to the *ver* family from which the PfEMP1 is transcribed, the VESA-1 resembles the PfEMP1 in more than one way (Baruch et al., 1995). Reportedly these share some similarity in peptide level as VESA-1 has a small cystine and lysine rich domain near its N-terminal region that closely resembles the cystine rich interdomain region of PfEMP1, reportedly involved in interaction with CD36 (Al-Khedery and Allred, 2006, Xiao et al., 2010) .Though CD36 was not found involved in the altered adhesion of *Babesia bovis* infected cells, but the similarity of the VESA1 to the PfEMP1 can be a probable indication of its role in altered cytoadherence in such infected cells.

1.4.2.2 Physiological alterations: Novel permeation pathways

Once inside the erythrocyte, the *Babesia* spp. feed on the host haemoglobin to meet the nutritional requirements (Rudzinska, 1976, Barry, 1982, Commins et al., 1988). But unlike in *Plasmodium* spp., the mechanism for uptake or for the degradation of haemoglobin in *Babesia* is not well understood (Rudzinska, 1976, Aikawa et al., 1966, Barry, 1982, Commins et al., 1988). In *Babesia* spp., no structure resembling typical cytostome or tubular structure had been reported (Rudzinska, 1976) nor do they feed on haemoglobin by phagocytosis(Rudzinska, 1976); they do not produce a typical food vacuole and haemoglobin metabolism here is devoid of any residual

haemozoin pigment formation (Langreth, 1976, Rudzinska, 1976, Rudzinska et al., 1983, Rudzinska et al., 1976, Kawai et al., 1999, Richier et al., 2006, Uilenberg, 2006). These facts indicate towards the presence of a rather highly efficient mechanism for internalization and degradation of haemoglobin in *Babesia*.

Over the years, extensive research with different strains of *Babesia* has established the involvement of different proteolytic enzymes in the degradation of the host cell haemoglobin as a part of nutrient acquisition of the parasite (Barry, 1982, Commins et al., 1988, Wright and Goodger, 1973, Wright et al., 1989, Mesplet et al., 2010). The process of haemoglobin degradation was reportedly slower when compared to the haemoglobin degradation in *Plasmodium berghei*, presumably indicating the possibility of a more thorough removal of haemoglobin-breakdown products in *Babesia* infected cells (Barry, 1982). Another hypothesis advocates about the presence of a fundamentally thorough haemoglobin degradation pathway in *Babesia* in comparison to the *Plasmodium* (Rudzinska, 1976, Simpson et al., 1967).

For the growth and development inside the erythrocyte these parasites need and eventually acquire, significant amount of metabolic precursors like sugars, purines and other micro-nutrient (Alkhalil et al., 2007). These molecules are limited in supply within the erythrocytes but are present in abundance in the extracellular milieu. These compounds however have significantly low intrinsic permeability across the erythrocyte membrane (Alkhalil et al., 2007). The metabolic wastes generated inside the erythrocyte need to be eliminated from the cell as well. These call for some modifications of the erythrocyte membrane and so are reportedly induced by the *Babesia*; similar in line to the NPP found in *Plasmodium* infected erythrocytes (Alkhalil et al., 2007, Desai et al., 1993).

With radioactive glucose and uptake assays with bovine erythrocytes it had been demonstrated that a) a marked increase in the uptake of glucose upon infection by *Babesia bovis*, b) a saturation point, that is quite similar to that of PSAC (of *Plasmodium*) (Alkhalil et al., 2007) and c) an effective inhibition of such uptake with phloridzin at a concentration similar to that known for PSAC (Upston and Gero, 1990, Alkhalil et al., 2004, Desai et al., 2000). These findings were reasoned to be indicative of the fact that the altered permeability in *Babesia* infected cells might be achieved by activating ion channels similar to the ones known in *Plasmodium*. (Alkhalil et al., 2007, Desai et al., 2000). However in parallel nucleoside uptake in *Babesia bovis* infected

erythrocytes was found to be a saturable process with and with distinct chemical reactivity (Gero and O'Sullivan, 1990). Another interesting study, conducted by Baumeister and colleagues reported uptake of a drug Fosmidomycin into *Babesia divergens* infected erythrocytes (Baumeister et al., 2011).

The molecular details of the pathways, ultimately leading to induction of the NPP in *Babesia* infected erythrocytes, are not properly known. But presumably these are by-and-large similar in nature to those reported in *Plasmodium* and involve transport proteins located onto the RBCM (Baumeister et al., 2003, Baumeister et al., 2006). These proteins presumably mediate the flux across the erythrocyte membrane or are involved in the activation of erythrocyte endogenous transporters through the actions of other activator proteins (Staines et al., 2007, Derbyshire et al., 2008), phosphorylation (Egee et al., 2002, Merckx et al., 2009, Slavic et al., 2009) and/or other stress factors (Huber et al., 2002).

1.4.3 Invasion induced compartmentation: *Babesia*

The invasion by *Babesia* spp. is a process, quiet similar to that of other Apicomplexan, pertaining basic steps of initial weak surface recognition and adhesion followed by reorientation of the parasite anteriority subsequent penetration (Lobo et al., 2013, Montero et al., 2006, Yokoyama et al., 2006). But unlike *Plasmodium*, *Babesia* can directly infect erythrocytes and complete their asexual developmental phases inside the cell. This indicates the possibility of a rather specific array of receptor–ligand interaction, during such invasion. Several proteins encoded by the parasite had been described to mediate the primary attachment initiating these adhesive interactions (Dubremetz et al., 1998, Zintl et al., 2002a, Yokoyama et al., 2006).

Species specific GPI anchored proteins, broadly termed as Merozoite Surface Antigens, including the *Babesia bovis* Variable Merozoite Surface Antigen (VMSA-1) and a 37 kDa *Babesia divergens* merozoite surface protein Bd37, are reported to be involved as parasite ligands during the invasion (Hines et al., 1992, Jasmer et al., 1992, Suarez et al., 2000, Delbecq et al., 2002).

Like other Apicomplexans, proteins secreted from the apical complex (rhoptries, micronemes and dense granules) located at the anterior end of the *Babesia* spp are reportedly involved in the events leading to invasion (Lobo, 2005). Of these proteins, the involvement of Rhoptry

Associated Proteins (RAP) [reviewed by (Yokoyama et al., 2006)], Apical Membrane Antigen 1 (AMA-1) and Thrombospondin-Related Anonymous Protein (TRAP) is most well understood (Lobo et al., 2013, Montero et al., 2009, Gaffar et al., 2004b, Gaffar et al., 2004a).

Proteins from the apical complex in general, play significant role in the cellular attachment, internalization and subsequently in the multiplication of the Apicomplexan parasites inside the host cell (Dubremetz et al., 1998, Hodder et al., 2001) *Babesia bovis* AMA1 is implicated in the erythrocyte invasion by the parasite whereas *Babesia divergens* AMA1 has been shown involved in forming and maintaining the 'moving junction' during the invasion (Montero et al., 2009, Yokoyama et al., 2006). An approximately 60kDa Rhoptry Associated Protein 1 (RAP-1) has been reported to be involved in erythrocyte invasion by *Babesia bovis* and *Babesia bigemina* and the RAP-1 encoding gene had been identified in *Babesia canis*, *Babesia ovis* and *Babesia divergens* as well (Suarez et al., 1991b, Suarez et al., 1991a) (Dubremetz et al., 1998, Yokoyama et al., 2006) implicating the importance of the role played by this ligand in invasion (Suarez et al., 1994)

A micronemal protein, Thrombospondin-Related Anonymous Protein (TRAP) is known to be secreted only during the erythrocytic stages of the development of *Babesia* and reportedly has analogy to their closely related counterparts in *Plasmodium falciparum* (Lobo et al., 2012, Lobo et al., 2013, Montero et al., 2009, Gaffar et al., 2004a, Gaffar et al., 2004b).However the precise role of TRAP in the erythrocyte invasion by *Babesia* needs further confirmation.

Independent studies have established sialic acid residues, sulphated glycosylaminoglycans, protease sensitive proteins of the erythrocyte surface and proteins of the Glycophorin family to be involved in and as receptors during the invasion (Kawai et al., 1999, Yokoyama et al., 2006, Cursino-Santos et al., 2014b, Lobo, 2005). But the molecular details underlining the receptor-ligand interactions leading to the erythrocyte invasion by *Babesia* remain largely unclear (Kawai et al., 1999, Dubremetz et al., 1998, Zintl et al., 2002a, Zintl et al., 2002b, Yokoyama et al., 2006).

Though the molecular details of the interaction underlying the erythrocyte invasion by the *Babesia* spp. are rather unclear but the choice of surface protein receptors and ligands for the invasion seem to be fairly similar to those known for *Plasmodium* spp (Cursino-Santos et al., 2014b, Lobo, 2005). It had been also demonstrated that neuraminidase sensitive receptors play

crucial role in the initiation of the invasion procedure by *Babesia bovis* and *Babesia divergens* (Gaffar et al., 2003, Zintl et al., 2002a). Lobo and colleagues (2005) demonstrated that, like *Plasmodium falciparum*, *Babesia divergens* too exploits Glycophorin A and B as receptors during their erythrocyte invasion and this similarity in turn indicates some conservation in the invasion mechanisms itself (Lobo, 2005).

During invasion *Babesia* forms a parasitophorus vacuole, much like *Plasmodium* and remains transiently encapsulated in it. However the PV is soon lost after the disintegration of the PVM and the parasite comes in direct contact with the cytosol (Rudzinska, 1976, Repnik et al., 2015). Spherical body', a *Babesia bovis* equivalent of *Plasmodium falciparum* 'Dense granules' are believed to be involved in the lysis of the PV and bringing these parasite in direct contact with the erythrocyte cytosol (Hines et al., 1995).3 proteins of the *Babesia bovis*-spherical body had been well characterised and they are found to be released inside the cytoplasmic phase of the infected erythrocyte during the later stages of infection(Hines et al., 1995, Ruef et al., 2000, Dowling et al., 1996). However contributions part of the parasite and the erythrocyte, towards the formation of the PVM is not completely understood.

1.5 Two parasites one host cell:

Limitations in metabolic resources coupled with a compact cytoskeleton, hindering host cell invasion, render the erythrocyte to be a choice of host cell for only a few parasite including the ones of genera *Plasmodium* and *Babesia*. The choice of erythrocyte by these obligate intracellular auxotrophic parasites, as host, reflects a higher level of adaptation on part of these parasites to this unusual envioronment. Different experimental evidence suggests that both of these parasites alter the erythrocyte and the intracellular environment to varied extent in order to customise it for their specific need. In the complete absence of any biosynthetic machinery and sub-cellular trafficking pathways in the erythrocyte, presumably these processes are majorly parasite driven. However the contribution on part of the erythrocyte components remains fairly unclear.

Over last few decades our understanding about the contribution of the erythrocyte during invasion and in invasion induced alterations, has also undergone major change. PVM, a major invasion induced compartment secludes the parasite temporarily or permanently from the host cytosol. For long time the erythrocytes contribution towards the formation of PVM had remained

obscure. Initial experiments with erythrocytes labelled with fluorescent lipid analog followed by their invasion with *Plasmodium falciparum*, had demonstrated incorporation of such lipid to the PVM of *Plasmodium falciparum* whereas RBCM proteins were reportedly found excluded (Ward et al., 1993): This finding was reasoned to be a suggesting an active prevention of internalization of such proteins by the invading parasite and the organization of these proteins with erythrocyte cytoskeleton as the determinant of the inclusion or exclusion [Review in (Lingelbach and Joiner, 1998)]

Over the last decade, insurgence of the concept of microdomains in the plasma membrane of cells, including in erythrocytes (Lingwood and Simons, 2010) led to re-examination of the *P. falciparum* infected erythrocyte and analysis, in terms of their PVM constituents(Murphy et al., 2004). These experiments further confirmed the absence of any erythrocyte cytoskeleton or cytoskeleton-associated proteins from the *P.falciparum*-PVM whereas suggested presence of a varied set of proteins associated with such microdomains or otherwise (Murphy et al., 2004, Lauer et al., 2000, Bietz et al., 2009).

Reportedly *P. falciparum* and *B. divergens* both use similar receptor-ligand interaction during the invasion, (Lobo, 2005) but the formation of the PVM in *B. divergens*-iRBC had not been well understood in the lack of any marker protein for the PVM following the formation and fate of PV had also remained equally difficult.

If the erythrocyte biophysical properties govern the recruitment of specific erythrocyte membrane components to the newly formed vacuolar compartments of the invading *Plasmodium falciaprum*, this recruitment should be generic amongst the parasites of erythrocyte. Therefore conclusively these would reflect the erythrocyte contribution towards this infection induced alteration (PV) and these proteins will therefore stand the chance to be used as a marker for PVM for studying its formation and fate by immunocytochmistry.

Additionally as the effects of host cell genetic programme on host-parasite interaction are minimal in case of RBC and its parasites, we believe that these parasites could also broaden our understanding of the various inducible physiological processes, generally silent in these genetically quiescent cells.

1.6 Objective

Plasmodium is known to cause major alterations inside the erythrocyte (Maier et al., 2009) and from the various reports available it seems, *Babesia* as well causes structural and physiological alterations to the erythrocyte. In a genetically and metabolically reduced cell like erythrocyte, any major structural or physiological alterations are presumably driven, to a great extent by the parasite encoded factors whereas the contribution of the host cell, at least in respect to erythrocyte, towards such alterations remains unclear at this moment.

In this study I aimed to the advantage of a *Babesia divergens* strain, adapted to human erythrocyte and performed a comparative analysis with *Plasmodium falciparum* maintained in identical erythrocytes, to elucidate and the contribution of the host erythrocyte in the infection induced structural alterations.

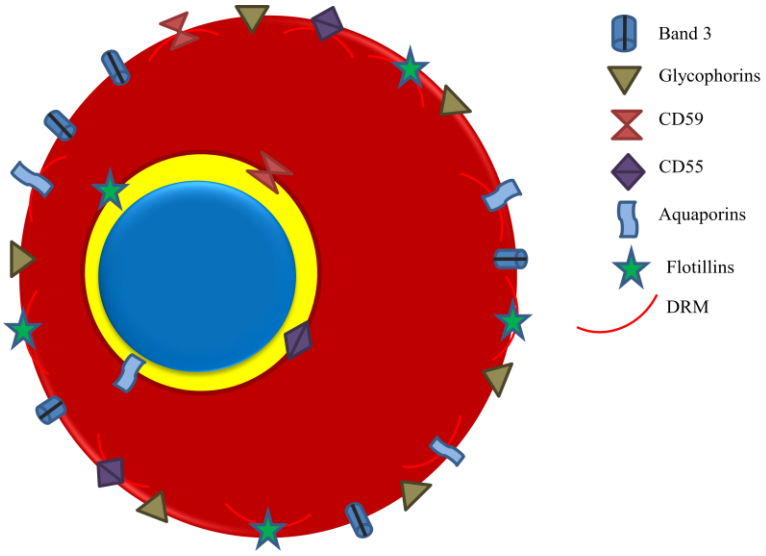


Figure 1.7 Recruitment of erythrocyte membrane proteins onto the PVM during the asexual blood stages of *Plasmodium falciparum*

GPI anchored and erythrocyte detergent resistant membrane micro domain associated proteins Flotillin-1, 2, CD55, CD59; and membrane spanning protein Aquaporin-1 and 3 was found recruited to the newly formed PVM whereas integral membrane and high copy number proteins Band3 and Glycophorin was found not recruited. Lauer et al, 2000, Murphy et al, 2004, Murphy et al, 2007, Bietz et al, 2009

I took help of electron microscopy to elucidate the ultrastructure of the resident parasites and also of the vacuolar compartments hosting them. I did a lipid labelling on erythrocyte membrane and thereafter followed it with invasion assay with *Plasmodium falciaprum* and *Babesia divergens* and time course microscopy to understand the involvement of erythrocyte membrane

lipids in the PVM of these two parasites. Additionally this technique helped me in elucidating the formation and maturation of the PVM in *Babesia divergens*.

Thereafter I chose a strict selection of erythrocyte membrane proteins (GPI anchored, membrane spanning, cytoskeleton associated, cytoskeletal and surface receptors) and performed immunofluorescence analysis, chemiluminescence analysis, biochemical analysis, to observe the presence /absence of these erythrocyte components on the vacuolar compartments generated during the invasion by these two related apicomplexa. The commonness in the recruitment of these components therefore could be ascribed on properties of the erythrocyte and any uniqueness presumably specific to the parasite

2 Materials and methods

2.1 Materials

2.1.1 Instrumentation

Instrument	Manufacturer
Analytical balance 2414	Sartorius
Autoclave (Varioklav)	Thermo scientific
Biofuge fresco centrifuge	Heraeus
Blotting apparatus	Phase
Film (X-ray) developing cassette	Rego
Ice machine (AF-20)	Scotsman
Incubator B5060-EC/CO_2	Heraeus
Incubator C16	Labotect
Laminar flow Herasafe	Heraeus
Mikro 22R refrigerated centrifuge	Hettich
Magnetic bubbler Combimag RCH	IKA
Magnetic columns	Miltenyi Biotech
Micro 22R refrigerated centrifuge	Hettich
pH meter	Greisinger electronic GmbH
Power Pac basic power supply	Biorad
Precision balance 1205 MP	Satorius
Roller mixer	NeoLab
Thermoblock	Stuart Scientific
Thermomixer 5436	Eppendorf
VARIO Mac separator	Miltenyi Biotech
Vista vision microscope	VWR
Vortexer vortex-genie 2	Scientific industries
Water bath	Köttermann
5810 R refrigerated centrifuge	Eppendorf

2.1.2 Utilities

Material	Provider
Centrifuge tubes PC	Beckman
Cryotubes	Starstedt
Culture flasks (25 cm^2 and 75 cm^2)	Greiner
Exposure cassettes	Rego
Glassware	Schott
Medical X-ray films RX NIF	Fuji
Microscope slides	VWR
Microscope cover slides, 24 x 60mm	Marienfeld Lab Glassware,Precision
Nitrocellulose membrane	Schleicher & Schuell
Pasteur pipettes	Brand GmBH
Petri dishes	VWR/ Greiner
Pipette tips	Sarstedt / Greiner

Plastic materials	Sarstedt / Eppendorf / Greiner
Reaction tubes (0.2 ml, 0.5 ml, 1.5 ml, 2.0 ml, 15 ml, 50 ml)	Sarstedt / Eppendorf
SDS sample loading tips	VWR
Whatman paper	Schleicher & Schuell

2.1.3 Chemicals /reagents

Chemicals	**Provider**
Acetone	Roth
Ammonium hydroxide (NH4OH)	Roth
Ammonium peroxydisulphate (APS)	Roth
Ammonium sulphate ($(NH_2)_2SO_4$)	Roth
Bovine serum albumin fraction V (BSA)	PAA
Bromo-phenol blue	Amersham Biosciences
Calcium chloride ($CaCl_2$)	Roth
Chloroform ($CHCl_3$)	Roth
Citrate phosphate dextrose solution (CPD)	Sigma
d-Glucose	Roth
d-sorbitol	Roth
Dimethyl sulphoxide (DMSO)	Roth
Dipotassium phosphate (K_2HPO_4)	Roth
Disodium phosphate (Na_2HPO_4)	Roth
1,4-dithio-DL- threitol (DTT)	Fluka
Ethanol p.a. (EtOH)	Roth
Ethylendiamintetra-acetic acid (EDTA)	Roth
E64D	Sigma-Aldrich
Glutaraldehyde	Roth
Glycine	Roth
Glycerol anhydrous	Applichem
HEPES	Applichem
Hoechst 33258	Molecular probes
Hydrochloric acid 37% (HCl)	Roth
Hydrogen peroxide (H_2O_2)	Merck
Isopropanol	Mercl
Luminol	Applichem
Magnesium chloride ($MgCl_2$)	Roth
Magnesium sulphate ($MgSO_4$)	Roth
Methanol	Roth
NNN′N-tetra methylene ethylene diamine (TEMED)	Roth
o-cresolsulfonephthalein (Cresol Red)	Sigma-Aldrich
p-Coumaric acid	Roth
PageRuler Prestained Protein Ladder	Thermo Scientific
Paraformaldehyde	Roth/Sigma-Aldrich
PKH26	Sigma-Aldrich
Potassium acetate (CH_3COOK)	Applichem

Potassium chloride (Kill)	Roth
Potassium dihydrogenphosphate (KH_2PO_4)	Roth
Phenylmethylsulfonylfluoride (PMSF)	Serva
Ponca red S Pure	Roth
Protease Inhibitor Cocktail Set III	Calbiochem
Proteinase K	Applichem
Rhodamin labelled Wheat germ agglutinin	Vector laboratories
Rotiphorese ®Gel 30	Roth
Saponin	Roth
Skimmed milk powder	Roth
SOB – medium	Roth
Sodium acetate (CH_3COONa, NaOAc)	Roth
Sodium carbonate (Na_2CO_3)	Roth
Sodium chloride (NaCl)	Roth
Sodium dodecyl sulphate (SDS)	Applichem
Sodium hydroxide (NaOH)	Merck
Sodium sulphite (Na2SO3)	Roth
Spectra multicolour broad range protein ladder	Fermentas
Sucrose	Roth
Sulpho_NHS_S-S_biotin	Sigma-Aldrich
Synthetic Band 3 peptide (ab177142) For Blocking Abcam Rabbit polyclonal Band 3-N terminus antibody	Epitomics (formerly Abcam)
Trichloroacetic acid ($CHCl_3$)	Roth
Tris	Applichem
Triton-X-100	Roth
Water (sterile, pyrogen-free)	Roth
1,4-dithio-DL-threitol (DTT)	Roth

2.1.4 Cell culture materials

Materials	**Provider**
AlbuMAXII	Invitrogen
D-Sorbitol	Roth
Gelafundin	B. Braun
Gentamycin	PAA
Giemsa	Merck
Human erythrocyte concentrate A Rh+	Blood bank, University Hospital Marburg
Human Plasma A Rh+	Blood bank, University Hospital Marburg
Hypoxanthine	CC Pro
Neomycin	Sigma
RPMI 1640	Gibco
RPMI 1640	PAA
WR99210	Jacobus Pharmaceuticals

2.1.5 Antibodies

Materials	Provider
Primary antibodies	
Mouse α-Hs Glycophorin A (Monoclonal)	Sigma-Aldrich, G7900, Clone E4
Rabbit α-Hs Aquaporin 1(Polyclonal)	Sigma-Aldrich, HPA019206
Rabbit α-Hs Aquaporin 3 (Polyclonal)	Sigma-Aldrich, SAB200111
Rabbit α-Hs Band 3 N-terminal (Monoclonal)	Abcam, Catalog No.3637-1
Rabbit α-Hs CD59 (Polyclonal)	Sigma-Aldrich, HPA026494
Rabbit α-Hs Flotillin-1(Polyclonal)	Sigma-Aldrich, HPA001393
Rabbit α-Hs Flotillin-2 (Polyclonal)	Sigma-Aldrich, HPA001396
Rabbit α-Hs Spectrin α, β (Polyclonal)	Sigma-Aldrich, S1515
Rabbit α-*Pf* EXP-1 (Polyclonal)	AG Lingelbach
Zebrafish α-Hs Band 3 C-terminal (Monoclonal)	AG Lingelbach
Secondary antibodies	
Goat α-mouse-Cy2	JacksonImmuno Research Laboratory
Goat α-mouse HRP	DAKO
Goat α-Rabbit-Cy3	Jackson Immuno Research Laboratory
Goat α-Rabbit -Cy5	Jackson Immuno Research Laboratory
Goat α-Rabbit HRP	DAKO

2.1.6 Cells and organisms

Strain	Genotype	Reference
***Plasmodium falciparum* clone 3D7**	The clone was obtained by limiting dilution of the original NF54 (Isolated near Schipol Airport, Amsterdam, Netherlands)	(Walliker et al., 1987)
Babesia divergens	Isolates from Rouen 1986	(Gorenflot et al., 1991)

2.1.7 Buffers and solutions for protein biochemistry

Buffers and solutions	Composition
Ammonium peroxisulfate (APS)	10% in double distilled H_2O
Blocking solution- IFA	3% bovine serum albumin in PBS pH 7.4
Blocking solution-Western blot	5% skim milk powder in PBS pH 7.4 + 0.05% Tween-20
Developer (X-Ray film)	6.4 mom Metol 80 mom Hydroquinone 571 mom sodium sulphite (Na_2SO_3) 452 mom sodium carbonate (Na_2CO_3) 34 mom Potassium bromide (KBr)
ECL developing solution	5 mom luminol 0.8 mom p-coumaric acid 200 mom Tris-HCl pH 8.5

Electrophoresis buffer (5X)	124 mom Tris 960 mom glycine 0.5 % SDS
Fixation buffer-IFA	4% Paraformaldehyde 0.0075% Glutaraldehyde In PBS pH 7.4
Fixation buffer1 –EM	8% Paraformaldehyde In 200mM HEPES
Fixation buffer2 –EM	8% Paraformaldehyde 0.015% Glutaraldehyde In 200mM HEPES
Fixation buffer3 –EM	X% Glutaraldehyde In 200mM HEPES
HEPES buffer-1	200mM HEPES
Permeabilisation buffer -IFA	0.1% Triton-X-100 in PBS pH 7.4
Phosphate Buffer Saline (PBS)	140 mom Sodium chloride (NaCl) 2.7 mom Potassium chloride (Kill) 1.4 mom mono-potassium phosphate (KH_2PO_4) 0.8 M did-sodium phosphate (Na_2HPO_4)
Ponca S staining solution	0.2% Ponca-S 3% trichloro acetic acid In double distilled water
Quenching buffer-IFA	125mM glycine in PBS pH7.4
SDS sample buffer (2X)	100 mom Tris/HCl pH 6.8 5mM EDTA 20% Glycerol (v/v) 4% SDS 0.2 % bromophenol blue 100 mom DTT
Solubilisation buffer	2% Triton-X-100 150mM Sodium chloride (NaCl) 2mM EDTA 100mM Tris/HCl pH 7.5
Separating buffer (4X)	1.5 mom Tris-HCl pH 8.8 0.4% SDS (w/v)
Stacking buffer (4X)	500 mom Tris-HCl pH 6.8 0.4% SDS (w/v)
Western blot transfer buffer	48 mom Tris-HCl pH 9.5 39 mom glycine 0.04% SDS (w/v) 20 % MetOH (v/v)
Western Blot wash buffer 1	TRIS-saline+0.1%Triton_X_100+0.05% Tween-20
Western Blot wash buffer 2	TRIS-saline+0.1%Triton_X_100
Western Blot wash buffer 3	TRIS-saline

2.1.8 Cell culture solutions

Freezing solution	**28 % glycerol (v/v)** **3% d-Sorbitol (w/v)** **0.65 % sodium chloride (NaCl)**
Thawing solutions	1. 12 % sodium chloride (w/v) solution 2. 1.6 % sodium chloride (w/v) solution 3. 0.9 % sodium chloride (w/v) solution
WR 99210	20 mom stock (8.6 mgs/1 ml DMSO) diluted to a working concentration of 20 µM with RPMI 1640 Final working concentration 5 nM

2.2 Methods

2.2.1 Cell culture: *Plasmodium falciparum* 3D7 and *Babesia divergens*

Asexual phases of the obligate intracellular apicomplexan, *Plasmodium falciparum* 3D7 and *Babesia divergens* (isolate Rouen 1986) (Gorenflot et al., 1991) were cultured in-vitro, in human erythrocytes by following the description of Trager and Jensen (Jensen and Trager, 1977). The cultures were maintained at a haematocrit of 4%. Human erythrocytes from blood group A, Rh +ve donors were obtained from the blood bank facility of Marburg University Clinic. The parasite cultures were maintained in RPMI media, supplemented with 10% human serum, 200 µM hypoxanthine and 0.1mg/ ml neomycin. The flasks containing these suspension cultures were maintained at a constant temperature of 37^{o}C with steady supply of a gas mixture containing 90% N_2, 5% of O_2 and CO_2 each.

Growth and differentiation of the parasites were monitored by regularly checking the parasitemia of the culture. Parasitemia of a particular culture is the ratio of iRBC to that of the total number of RBC present, expressed as a percentage factor. For checking parasitemia, on clean grease free slide, a thin smear with approximately 6µl of iRBC (from 35 ml of *P. falciparum* and 25 ml of *B. divergens* culture respectively) was made and it was air dried. The smear was then fixed with 100% methanol for 30 seconds and was stained with Giemsa's stain for 10-15 minutes.

The excess stain was washed off and the slide was observed under the 100x oil immersion objective of a light microscope. For subsequent cell culture, the parasitemia was determined by averaging at least 3 independent counts of three different areas on the smear. Once the parasitemia was determined, the culture was either continued on with or was sub-cultured.

In an *in vitro* culture set up, *Plasmodium falciparum* can grow up to the parasitemia of 10% (of the trophozoites stages) but beyond this point they do not remain healthy whereas *Babesia divergens* can reach up to a parasitemia of 70%-80% and can still remain healthy. The development of *Plasmodium falciparum* is completed in 48 hours and is characterised by alternative division and differentiation cycles giving rise to morphologically distinct developmental stages. In around 9 hours of the development cycle, the *Babesia divergens* undergoes differentiation followed by nuclear division, giving rise to up to 4 infectious daughter cells. However the initial stages after differentiation are not morphologically distinguishable from each other, whereas the post nuclear division stages have unique morphology.

2.3 Parasitemia and subculture of *Plasmodium*

Given the facts, a) 1ml of the erythrocyte concentrate (EC) used in the parasite culture contained, 1×10^{10} RBCs b) alternating division and differentiation cycles are characteristic to *Plasmodium falciparum* and c) *Plasmodium falciparum* did not stay healthy beyond the parasitemia of 10% of the trophozoite stages in culture, a 5 day cell culture plan was designed. It started with 4×10^{7} parasites (0.4% parasitemia) in 1×10^{10} RBC (1ml of EC) to yield a final of 1×10^{9} parasites (10% of parasitemia in the total volume of RBC (1×10^{10} RBC) present in culture at the end of the 5^{th} day.

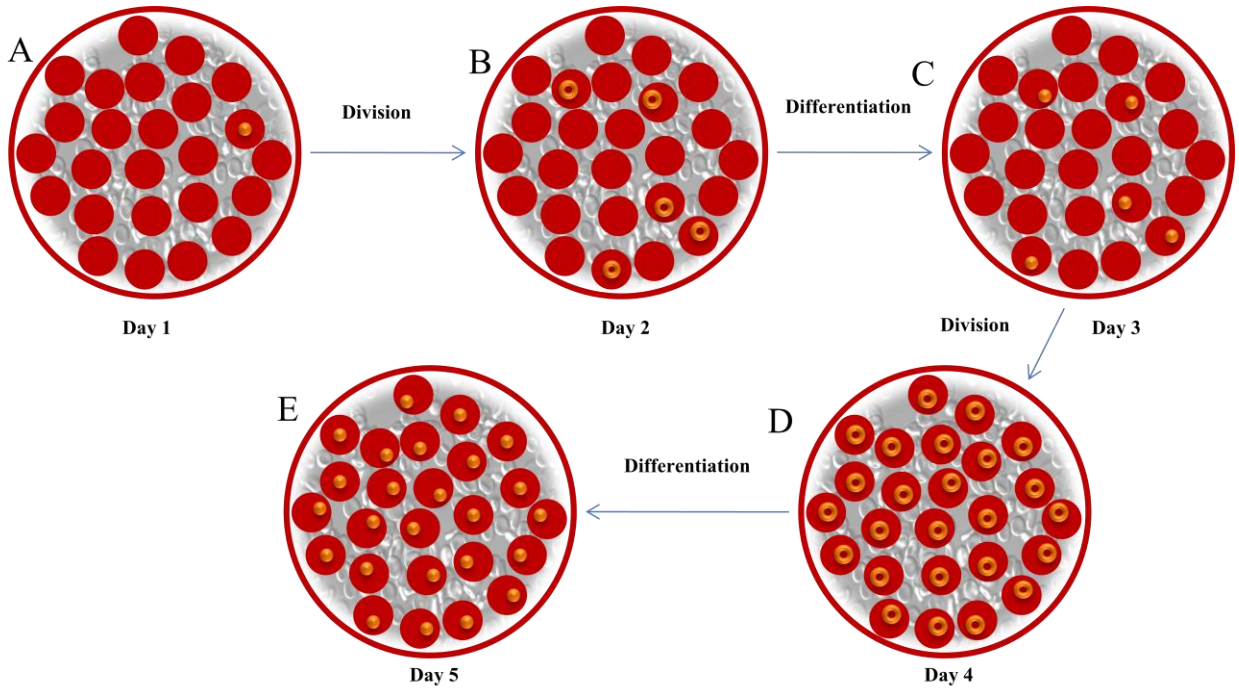


Figure 2.1: *In vitro* development and asexual stages of *Plasmodium falciparum*: a relative idea of parasitemia with 25 RBCs (from a total of 250 RBCs present in this culture)

A) **Day 1**: Single trophozoite infects single RBC; Parasitemia (1/250) x 100=0.4% **trophs**. The trophozoites would grow up to schizont stages and then schizonts will divide to give rise to ring, 5 times the initial number of trophs.

B) **Day 2**: 5 rings are developed from the single trophozoite; Parasitemia (5/250) x 100= 2% **rings** these rings would differentiate to give rise to same number of trophozoites.

C) **Day 3**: 5 trophozoites are formed after differentiation and development of the 5 rings. Parasitemia (5/250) x 100=2% **trophs**. These trophozoites will grow further to become schizonts and thereafter divide to give rise to 25 rings; 5 times of the initial number of the trophozoites present at that time.

D) **Day 4**: 25 rings developed after division of the 5 trophozoites present in the culture, Parasitemia of (25/250) x 100=10% **rings**. The parasites will grow and further mature into trophozoite stages without any cell death.

E) **Day 5**: 25 trophozoites result from the development of the 25 rings present on the previous day. Parasitemia of (25/250) x 100=10% of trophs. *Plasmodium falciparum* did not stay healthy beyond this point in culture; hence the same culture was not continued beyond this point. The cultures were split to start a fresh subculture from point A.

2.3.1 Synchronisation of *Plasmodium falciparum*

I synchronised the parasites in culture, routinely, by a physical method of applying a high gradient magnetic field (Paul et al., 1981) or by chemical methods like Gelafundin floatation (Pasvol et al., 1978) and/or Sorbitol treatment (Lambros and Vanderberg, 1979).

2.3.1.1 Synchronisation of *Plasmodium falciparum* using a high gradient magnetic field (Paul et al., 1981)

A physical and unobtrusive method for the enrichment of *P. falciparum*, towards its trophozoite stage is by applying a high gradient magnetic field and arresting such trophozoite stages inside the a ferromagnetic column (Paul et al., 1981). During its intra-erythrocytic development, *Plasmodium falciparum* feeds on the haemoglobin present in the cell cytosol. It digests the haemoglobin (Fe^{++}), a diamagnetic compound, utilises the peptic digest products while releasing the indigestible Haem fraction. This Haem polymerizes and forms Haemozoin (Fe^{+++}), a paramagnetic compound. Over time Haemozoin is accumulated inside the food vacuole of the parasite. By applying a very high magnetic field and exploiting the paramagnetic nature of the Haemozoin containing food vacuoles, parasites in their later stages can be enriched up to a very high concentration and thereafter can be isolated by (Paul et al., 1981).

The parasitemia of the culture to be synchronised, was maintained around 5% of trophozoites or beyond; and the parasitemia was checked by microscopy on Giemsa-stained slides .VarioMACS separator (Miltenyi Biotec) and suitable columns i.e. MACs-CS columns (Miltenyi Biotec) were used for the separation. The column was washed first with PBS (pH 7.4) followed by a wash with 3% BSA in PBS (pH 7.4). The parasite culture was then gradually added to the column.

When the column was fitted in the separator, the magnetic field was amplified manifolds and the ferromagnetic fibers present inside the column acted as a magnetic trap for the parasitized erythrocytes flowing through. These lead to a highly efficient trapping of the iRBCs containing later stages of the parasites inside, whereas the erythrocytes containing early stages of the parasite ran through without any obtrusion. After passing the entire culture through the column, pre-warmed PBS (pH 7.4) was passed through it, till elute was visibly clear. This step eliminated all of the non-infected erythrocytes stuck along the mesh.

The column was then removed from the VarioMACs separator to release it from the magnetic field and was fitted into a stand. The entrapped cells were eluted from the column, with pre-warmed PBS (pH7.4), till the final elute was opaque and was not red anymore. Eluates were pooled in and were thereafter centrifuged at 1600 x g for 2 minutes. The pellet was washed twice, with pre-warmed serum supplemented RPMI and then the parasitemia was checked. Generally by this process, around 70%-80% enrichment of the trophozoites could be achieved.

2.3.1.2 Synchronisation of *Plasmodium falciparum* via Gelafundin floatation (Pasvol et al., 1978)

Knobs are cup shaped electron dense protrusions, formed beneath the plasma membrane of *Plasmodium*-infected erythrocytes, during the later erythrocytic stages of (Luse and Miller, 1971). These knobs are moderators of the cytoadherence properties of these infected erythrocytes and are also involved in sequestering these cells in different organs of the body (Leech et al., 1984). Formation of knobs however changes the buoyancy of the respective erythrocytes and this altered buoyancy is exploited to isolate the later trophozoite stages of *Plasmodium falciparum*-iRBC by chemical treatment with Gelafundin (Pasvol et al., 1978).

A culture of *Plasmodium falciparum* (parasitemia of 5% trophozoites or more) was centrifuged at 1600 x g for 2 minutes and the supernatant was removed. The pellet was resuspended in 9 ml of Gelafundin solution. The pellet-Gelafundin suspension was allowed to incubate erect at 37^{o}C for 10 -15 minutes or till a visible demarcation between the opaque upper and denser lower layer of infected erythrocytes was achieved.

The increase in buoyancy caused by the knobs (*Plasmodium falciparum*-iRBC with later stages of parasite inside) rendered some of the infected cells incapable of sedimenting fast. Such cells were seen as the upper opaque layer, whereas the erythrocytes with other stages of infection sediment as usual. The upper opaque layer was carefully isolated and was centrifuged at 1600 x g for 2 minutes. This was followed by 2 washes with RPMI to remove any traces of Gelafundin. Thereafter the parasitemia of the pellet was determined. An enrichment of up to 50%-60% of the trophozoites could generally be achieved by this treatment.

2.3.1.3 Synchronisation of *Plasmodium falciparum* with Sorbitol treatment (Lambros and Vanderberg, 1979)

Intra-erythrocytic developmental of *Plasmodium falciparum* leads to alterations in the membrane permeability of these infected erythrocytes in the later stages of the infection (Alkhalil et al., 2007). An increase in the permeability of the RBC membrane allows a sugar alcohol Sorbitol to enter these cells and cause osmotic imbalance, ultimately resulting in haemolysis (Lambros and Vanderberg, 1979). Erythrocytes with early stages of *Plasmodium* spp. are less permeable to Sorbitol and can resist such imbalance whereas the later stages (trophozoites and schizonts) are killed. Such biochemical alteration of the infected erythrocyte is exploited to enrich the early

stages (rings) of *Plasmodium falciparum* by treatment with Sorbitol (Lambros and Vanderberg 1979).

The culture to be treated with Sorbitol was preferably maintained in its early ring stages (3%-4%). The culture was centrifuged at 1600 x g for 2 minutes. The pellet obtained was dissolved in 8 ml of 5% Sorbitol solution and was incubated at 37^{o}C for 10 minutes. Post-incubation the entire contents were centrifuged at 1600 x g for 2 minutes; the supernatant was discarded and the pellet was washed 2 times with RPMI to remove traces of Sorbitol. The total volume of the pellet was plated with 1 ml of EC and was incubated. After 1-2 hours of plating the cells, microscopy was done to test the success of the synchronisation.

2.3.2 Parasitemia and subculture of *Babesia divergens*

Unlike *Plasmodium* spp. the intra-erythrocytic developmental stages of *Babesia divergens* had not been synchronised. The natural progression of the infection is from invading merozoites to differentiated merozoite stages, and there is no nuclear division till this stage of the infection (Rudzinska et al., 1976, Repnik et al., 2015). The differentiated merozoites of *Babesia* spp. lose their PVM and nuclear division follows, giving raise to the trophozoite stages (Rudzinska et al., 1976). The trophozoites undergo nuclear division and morphological changes, giving rise to the piriform merozoite stages. The piriform merozoites are arranged in clusters of 2 or 4 parasites frequently referred to as Maltese cross (Rudzinska et al., 1976). Eventually these structures dislodge and the merozoites egress to infect fresh erythrocytes. *Babesia divergens* can reach a parasitemia of up to 80%-90% *in vitro* without significant haemolysis and/or parasites death.

Given these facts a simple cell culture plan to sustain the culture and to maintain the haematocrit at 4% was developed. Depending upon the parasitemia, 6-8 ml of old parasite culture (iRBC + RBC) was added to 800 µl of non-infected erythrocyte (EC) to which 25 ml serum supplemented RPMI was added and was incubated at 37^{o}C.

2.3.3 Synchronisation of *Babesia divergens*

Babesia divergens loses the PVM soon after the invasion and prior to any nuclear division (Repnik et al., 2015). For my experiments, I needed to study the components of the PVM in *B. divergens*; therefore I needed these ‘early’ pre-nuclear division stages. Moreover having more numbers of these ‘early’ stages of *Babesia* spp. was beneficial as it helped me quantify and

substantiate my observations. To maintain the parasites in uniform stages of development and also to get the developmental stages as per requirement, I attempted to synchronise the culture of *Babesia divergens*.

There is no prominent food vacuole formation in a *Babesia* spp.(Rudzinska, 1976) (Langreth, 1976) (Kawai et al., 1999) hence I could not achieve any enrichment by applying high intensity magnetic field. On the plasma membrane of *Babesia bovis*-iRBC, ridge like structures(Hutchings et al., 2007), akin to the knob like structures seen in *Plasmodium falciparum*-iRBC are reported (Luse and Miller, 1971). However my attempts to treat *B. divergens* with Gelafundin, known to exploit the knob formation and associated morphological alteration in *Plasmodium* spp,-iRBC failed as well. When I treated *Babesia divergens*-iRBC with 5% Sorbitol in a similar process like *Plasmodium* spp.-iRBC are treated, it failed to synchronise the *Babesia* culture. This was in spite of the fact that alteration of membrane permeability, akin to what was reported in *Plasmodium* spp.-iRBC, had also been reported in *Babesia* spp.-iRBC (Alkhalil et al., 2007). What I could maximally achieve was a transient enrichment of the early differentiated merozoite stages of *Babesia divergens,* by following a physical method of controlled contact between the non-infected erythrocyte and *Babesia divergens*-iRBC with a very high parasitemia (70%-80%).

2.3.3.1 Synchronisation of *Babesia divergens*: Transient enrichment of differentiated merozoites

A rough practice followed in our laboratory allowed a short and controlled contact between non-infected and *Plasmodium falciparum* infected erythrocytes [with high parasitemia of Schizonts (8%-10%)] to get freshly infected erythrocytes, required for some ‘time course’ experiments. I used the same technique with *Babesia divergens*-iRBC and non-infected RBC to get the earlier stages of infection of the parasite and then attempted to propagate them. The invasion rate was very low but the invasion was approximately simultaneous. I followed the invasion, by microscopy on these freshly infected erythrocytes after each 4 hours till 24 hours. I found that till around 8 hours (1 complete cycle) they were moderately synchronous but thereafter they lost synchrony. I made few alterations in the protocol, like adjusting the volume of non-infected to infected erythrocytes to the standard 4% haematocrit, changed the time of incubation to 30 minutes from 10 minutes followed in the procedure and altered the incubation temperature to 37^{o}C from incubation in room temperature as practiced. I found the infection rate was higher.

The synchrony lasted a few hours more till 10-12 hours (~2 cycles of the parasite development). Thereafter to get transient enrichment of the early stages of *Babesia divergens*-iRBC, I always followed this technique.

The parasite culture was centrifuged at 1600 x g for 2 minutes and the supernatant was discarded. On to the compact pellet 1000 µl of fresh erythrocyte was gently added without disturbing the pellet. It was allowed to incubate erect at 37^{o}C for 30 minutes. This is to ensure, at least some of the non-infected RBCs left on top gets infected by the *Babesia divergens* merozoites from the iRBC beneath. After incubation, 900 µl of blood from the top of this iRBC-RBC was carefully taken out without disturbing the pellet beneath. This was placed in a new plate with 25 ml of serum supplemented RPMI media and was incubated. The infection rate being very low (2%-5%) but moderately simultaneous, the parasites inside these iRBC were generally synchronous for around 1-2 cycles i.e. ~12 hours; after which the synchronicity was lost.

2.3.4 Cryopreservation of *Plasmodium falciparum* and *Babesia divergens*

The *in vitro* culture of *Plasmodium falciparum* was frozen when the parasitemia reached 5-10% of the ring stage. For *Babesia divergens*-iRBC, when the differentiated merozoites stages were around 50% of the total iRBC, the culture was frozen. The respective parasite culture was centrifuged at 1600 x g for 2 minutes and the supernatant was discarded. To the cell pellet equal volume of freezing solution (1:1) was added. The pellet was properly resuspended in the freezing solution prior to transferring it to a labelled cryo-tube and snap-freezing in liquid nitrogen. After around 10-15 minutes of incubation in the liquid nitrogen, the cryo-tubes with the frozen cells were finally stored in liquid nitrogen tank or at -80^{o}C freezer.

2.3.5 Thawing of cryo-preserved parasites: *Plasmodium falciparum* and *Babesia divergens*

To thaw the frozen parasites, the cryo-tubes were initially thawed in room temperature and their contents were then transferred to a 15 ml falcon tube. To this 200 µl of Thawing Solution I (containing 12% NaCl dissolved in sterile distilled water) was added drop-wise with gentle but thorough mixing. The tube was allowed to stand at room temperature for 3 minutes. After this incubation, 5 ml of Solution II (containing 1.6% NaCl dissolved in sterile distilled water) was

added with gentle mixing and the tube was incubated at room temperature, for 3 minutes. After this, 5 ml of Solution III (containing 0.9% NaCl dissolved in sterile distilled water mixed with 2% glucose dissolved in sterile distilled water) was added and the contents were mixed thoroughly. The tube was allowed to stand in room temperature for 3 minutes. Then the total content was centrifuged at 1600 x g for 2 minutes. The supernatant was discarded and the pellet was washed twice with pre-warmed RPMI to remove any traces of NaCl. The volume of the pellet was made up to 1000 µl with non-infected RBC and this was transferred to cell culture flasks. Media was added and was allowed to incubate.

Slides were made from day 2nd onwards to check parasite return. Once the parasites were visible and the parasitemia was above 2%, the parasites were sub-cultured.

2.3.6 Methods with non-infected erythrocytes

The aim of this thesis was to compare the constituents of the PVM formed during the invasion of Apicomplexan parasites *Plasmodium falciparum* and *Babesia divergens* in order to understand contributions on part of the erythrocyte and the parasite. Therefore the chemical units of a membrane i.e. the proteins and lipid components were analyzed using protein and lipid markers of the erythrocyte plasma membrane and their incorporation onto the PVM of the infected erythrocytes was followed with microscopy. Non-infected erythrocytes were used as positive control. For some of these studies non-infected erythrocytes were chemically treated with dyes or chemical adjuvant followed by parasite invasion and further biochemical analysis or immunodetection to study any invasion induced alteration in them.

2.3.6.1 Labelling of membrane lipids: PKH26-dye staining

The hypothesis in practice about the lipid constituents of the PVM in the Apicomplexan is that the erythrocyte and the parasite both contribute towards the lipid components in the newly forming PVM (Lingelbach and Joiner, 1998). However the majority of the work regarding the lipid constituents of the PVM is done on *Plasmodium falciparum*.[reviewed in (Lingelbach and Joiner, 1998)]. To study the incorporation of erythrocyte membrane lipids into the PVM of *Babesia divergens,* I carried out labelling of the erythrocyte membrane lipids by a non-transferable lipid analog-tagged with dye molecule. The labelling was followed by parasite invasion of the labelled cells and thereafter microscopy. I monitored and imaged live infected

and non-infected RBC to monitor the formation and disintegration of the PVM. I performed chemifluorescence analysis on fixed-permeabilized PKH26-Cy3 stained iRBC to study the recruitment of erythrocyte membrane lipids onto the PVM. I also performed IFA on fixed-permeabilized PKH26-Cy3 stained iRBC together with antibodies against erythrocyte membrane proteins, and detected them with Cy2 conjugated secondary to understand the co-localization of erythrocyte membrane proteins with respect to the membrane lipids onto the newly formed PVM of *Babesia divergens.*

Following the manufacturer's protocol, I adjusted this experiment for a volume of 5 x 10^8 cells, for the erythrocyte concentrate (EC) it was 50 µl (5 x 10^8). As the staining was suggested to be optimal for this volume, when I needed more cells, I increased the number of individual units. Apart the rolling-incubation steps all other steps were performed under sterile conditions in LAF chamber.

Under aseptic conditions 50 µl of EC in each vial/s were washed once with sterile serum supplemented RPMI followed by two washes with sterile PBS (pH 7.4). Thereafter the contents were centrifuged at 1600 x g for 2 minutes. To the final pellet obtained after the 3rd wash 250 µl of Diluent-C (provided in the kit), was added and was mixed gently. To this Diluent-C-RBC suspension 1 µl of PKH26-Cy3 conjugate was added and was mixed gently. The contents were incubated at room temperature with gentle rolling for 10 minutes. After 10 minutes of incubation, double the volume of the suspension in the vial [(Diluent-C, 250 µl) + (RBC, 50 µl) (PKH26, 1 µl) =250 µl; therefore 500ul, of 3% BSA in PBS (pH 7.4)] was added and incubated on roller for 5 minutes in room temperature. The contents of the vial were then centrifuged at 1600 x g for 2 minutes. The pellet was washed twice with sterile PBS followed by a final wash with serum supplemented RPMI. The cells were now prepared for further experiments.

2.3.6.2 Invasion assay of labelled erythrocytes

Each invasion experiment was carried out with the haematocrit calculation of small cell culture plate, i.e.500 µl of blood in 12 ml or 15 ml of serum supplemented RPMI media for *Babesia divergens* and *Plasmodium falciparum* respectively. Any fresh RBC added during the continuation of the culture was also labelled with PKH26-dye conjugate, to get the maximum possible results after the time of incubation.

For *Plasmodium falciparum*, 500 µl of PKH26-labelled RBC was incubated with *Plasmodium falciparum*-iRBCs, containing 1 x 10^8 of the trophozoites (volume was calculated from parasitemia) in 15 ml of RPMI. A maximum parasitemia of 10% of trophozoietes was aimed for (5 x 10^8 in this case), after 48 hours (a complete asexual developmental cycle of *Plasmodium falciparum* is completed in 48 hours).

For *Babesia divergens*, 500 µl of PKH26-Cy3 labelled RBC was incubated with 1 x 10^9 *Babesia divergens*-iRBCs (volume was calculated from parasitemia). The final parasitemia aimed for, after 48 hours of incubation, (same time of incubation like was followed for *Plasmodium falciaprum*) was roughly around 60%

2.3.7 Methods with infected erythrocytes

2.3.7.1 Biochemical methods

2.3.7.1.1 Fractionation of erythrocytes, infected with *Plasmodium falciparum* and *Babesia divergens*: Hypotonic lysis

Erythrocytes, with the infecting *Plasmodium falciparum* in trophozoite stages, were enriched approximately up to 65%-70% by Gelafundin floatation and/or by magnetic field based isolation technique. Erythrocytes infected with *Babesia divergens* were allowed to reach a similar parasitemia of approximately 65%-70%. To isolate the membrane fractions (required for immuno-blot analysis) of the respective cells, the infected erythrocytes in parallel to the non-infected erythrocytes were subjected to hypotonic lysis. 2 x 10^8 iRBC and RBC (each) were washed several times in PBS (pH 7.4) before resuspending in 1mM Tris (Stock: 10mM) supplemented with PIC and PMSF. This was subjected to a minimum of 3 cycles of freezing (by dropping in liquid nitrogen) followed by thawing. The contents were thereafter centrifuged at 36000 x g for 20 minutes at 4^{o}C. The pellet fractions represent the membrane components and this was isolated. The soluble fractions were centrifuged again at 36000 x g for 20 minutes at 4^{o}C to collect any remaining membrane fraction dissolved in it. The membrane fractions from these 2 steps were pooled in and were washed several times in PBS (pH 7.4) to remove residual haemoglobin before further work. The supernatant fractions represent the soluble erythrocyte proteins and cytosolic proteins from the parasite; these too were pooled in for further experiments.

The pellets obtained from RBC, *Plasmodium falciparum*-iRBC and *Babesia divergens*-iRBC was washed 4-6 times in PBS (pH 7.4) supplemented with PIC (1:200) and PMSF prior to boiling it in SDS sample buffer at 100°C for 10 minutes. The soluble fractions were also boiled in SDS sample buffer at 100°C for 10 minutes prior to SDS-PAGE analysis.

The treated samples were loaded on the freshly prepared, poly-acrylamide gel alongside a pre-stained protein marker (Thermo Scientific).

2.3.7.1.2 SDS-PAGEs (Laemmli, 1970)

Sodium dodecyl sulphate poly-acrylamide gel electrophoresis (SDS-PAGE) is widely used to separate individual proteins according to their charge and molecular weight in order to isolate them from a pull of proteins .When treated with a denaturing detergent SDS, the 3-dimensional structure of the protein is disturbed and an overall negative charge is ascribed onto the SDS-polypeptide complexes. Thereafter in an electric field, these negatively charged proteins move towards the positive pole and are separated according to their molecular weight, eliminating the differences between mass: charge ratios of the proteins. Additionally, boiling the protein samples in SDS-PAGE sample buffer (containing Dithiothreitol) minimises the formation of di-sulphide bridges thus supporting protein denaturation.

Based on the expected size of the protein of interest, the percentage poly-acrylamide present in the separating-gel was adjusted. Usually 10-12% acrylamide gel was used unless a low molecular weight protein was being studied; then the concentration was adjusted accordingly. The protein were run at 90 V to migrate in the stacking gel and once they entered the separating gel, the voltage was increased to 110 V. Protein ladder used to detect the molecular weight of the proteins were from Fermentas (Prestained Protein ladder).

Table 2.2.1: Pipetting scheme for a 12% gel.

Reagent	**Stacking gel**	**Separating gel**
Gel buffer	2.5 ml	7.5 ml
30% acrylamide	1.3 ml	12 ml
ddH20	6.2 ml	10.25 ml
APS	250 µl	200 µl
TEMED	15 µl	25 µl

2.3.7.1.3 Semi-dry immuno-blotting (Towbin et al., 1979)

To detect individual proteins separated by SDS-PAGE, immunodetection was performed with antibodies raised against the particular proteins. After the PAGE-separation the proteins were transferred from the gel matrix to a nitrocellulose membrane by applying an electric field.

For the transfer, the gel was removed carefully from the gel unit. The 'stacking' part of the gel was removed before gently placing the 'separating' part of the gel on a nitrocellulose membrane. This nitrocellulose membrane was cut to be of an exact size of the gel (this helped uniform transfer) and was soaked in transfer buffer (supplemented with 20% Methanol).Whatman filter papers, cut to the same size of the gel, were also soaked in the transfer buffer (supplemented with 20 % Methanol) and were placed as 3 on either side of the gel. This whole unit was transferred into a blotting chamber.

For a uniform transfer, air-bubbles formed and/or stuck inside this unit, were removed by gently pressing Whatman paper-Nitrocellulose membrane-Gel-Whatman 'sandwich' with a roller.

The transfer was carried out at a constant current of $1mA/cm^2$ for 1 hour and thereafter the 'sandwich' was dismantled. Once the membrane was removed, the transfer was confirmed by staining the membrane for 5 minutes with Ponceau red reagent and rinsing with distilled water till the transferred bands were visible.

This membrane was then blocked for an hour at room temperature in a solution of 5% skimmed milk dissolved in PBS (pH 7.4) supplemented with 0.05% Tween-20. After blocking the membrane against any non-specific antibody binding, the membrane was incubated with 1º antibody solution (diluted as per requirements) in 5% skimmed milk dissolved in PBS (pH 7.4) supplemented with 0.05% Tween-20. Incubation with 1º antibody was performed overnight at 4ºC on a roller. On the following day, the membrane was washed for a minimum of 3 times, 5 minutes each, to remove any unbound antibody. These 3 washes were carried out as follows: 1st wash was with TRIS-saline + 0.1% Triton-X-100 + 0.05% Tween-20; the 2nd wash was with TRIS-saline + 0.1% Triton-X-100 and 3rd wash was with TRIS-saline. After these washes, the membrane was incubated for 2 hours at room temperature in the 2º antibody solution, diluted in 5% skimmed milk dissolved in PBS (pH 7.4) and supplemented with 0.05% Tween-20. After this incubation step, the nitrocellulose membrane was washed for a minimum of 3 times, 5

minutes each to remove any unbound antibody. These 3 washes were carried out as were done to remove excess primary antibody.

The membrane was thereafter adjusted in the developing cassette, was incubated for 60 seconds in ECL solution (supplemented with 0.02% H_2O_2) and was exposed to the medical X-ray film in the dark room to detect the chemiluminescence. The X-ray was thereafter developed.

2.3.7.2 Fluorescence Microscopy

2.3.7.2.1 Live cell imaging

The invasion of PKH26-Cy3 labelled erythrocytes by *Babesia divergens* was monitored at 8 hours, 24 hours and 48 hours (post invasion) and by *Plasmodium falciparum*, was monitored at 24 hours and 48 hours (post invasion). PKH26-Cy3 labelled non-infected erythrocytes were used as positive controls. Erythrocytes were visualized using Zeiss Axio Observer inverse epifluorescence microscope system.

100-200 µl of the respective parasite culture was taken out, after mixing the plate well (to distribute the iRBC evenly (iRBC tend to settle at the bottom of the plate) and was centrifuged at 1600 x g for 2 minutes. The pellet was washed once with PBS (pH7.4) and was centrifuged again at 1600 x g for 2 minutes. This pellet was dissolved in PBS (pH 7.4) containing Hoechst 33258 (10ug/ ml) (PBS: Hoechst=1:5000) and was incubated for 2-5 minutes at 37^{o}C. A thin smear was made on either Concanavalin-A coated cover-slips or on clean slides under cover slips from Marienfeld Lab Glassware. The cells were observed with suitable filters under the Zeiss Axio Observer inverse epifluorescence microscope and were imaged with Axiovision 4 software. Exposure time was kept less than 95 milliseconds to eliminate risk photo-bleaching and cell lysis.

2.3.7.2.2 Immuno-fluorescence assay (Tonkin et al., 2004)

The immunofluorescence assays were performed following the protocol laid out by Tonkin and colleagues (2004). Erythrocytes infected with *Plasmodium falciparum* or *Babesia divergens* were enriched prior to fixing with 4% paraformaldehyde supplemented with 0.0075% glutaraldehyde dissolved in PBS (pH7.4) for 30 minutes at 37^{o}C with thorough mixing. Non-infected erythrocytes were treated similarly to be used as positive controls. The blackish-red pellet obtained after centrifuging the iRBC at 1600 x g for 2 minutes was permeabilized by

treating with quenching-permeabilising buffer for 15 minutes at room temperature with gentle rolling. This buffer contains Glycine at a final concentration of 125 mM in 0.1% Triton-X-100 in PBS (pH 7.4). The contents were centrifuged again at 1600 x g for 2 minutes. A compact pellet and a greenish supernatant is generally an indication of a successful treatment so far; whereas appearance of red trail of cells on the wall of the tube indicates lysis or improper fixation.

The cells were then blocked against non-specific binding for an hour at room temperature in 3% BSA in PBS (pH 7.4). These erythrocytes were incubated overnight at 4^{o}C in primary antibody dissolved in 3% BSA in PBS (pH 7.4). On the following day the primary antibody was removed and the cells were washed a minimum of 3 times for 10 minutes each in PBS (pH 7.4) with gentle rolling. Thereafter the cells were incubated for 2 hours at room temperature in secondary antibody with a Cy-2, Cy-3 or Cy-5 conjugated; dissolved in 3% BSA in PBS (pH 7.4). The secondary antibody was removed and the cells were washed a minimum of 3 times for 10 minutes each in PBS (pH 7.4) with rolling. Nuclear stain Hoechst 33258 (10ug/ ml) (PBS: Hoechst=1:50000) was added in the last wash step. A thin smear of the cells were made on either Concanavalin-A coated cover-slips or clean slides with cover slips from Marienfeld Lab Glassware. The cells were observed with suitable filters under the Zeiss Axio Observer inverse epifluorescence microscope fitted with Axiovision 4 software.

2.3.7.2.3 Immunofluorescence assay with 4% PFD only

For some initial experiments as an alternative to Tonkin's fixation procedure for RBC in suspension (4% paraformaldehyde supplemented with 0.0075% glutaraldehyde), the cells were fixed with 4% Paraformaldehyde alone. For this around 6 µl of RBC and iRBC was smeared on slides and were subjected to air drying. 4% paraformaldehyde was smeared on to the slides and the slides were incubated inside a moist chamber at room temperature for 30 minutes with gentle shaking. After the incubation, excess paraformaldehyde was removed and the slide was covered with a quenching-permeabilising buffer; containing Glycine at a final concentration of 125 mM in 0.1% Triton-X-100 in PBS (pH 7.4) and incubated in moist chamber for 15 minutes at room temperature, with gentle shaking. After the incubation the excess liquid was drained and the slides were air dried (under LAF) before subjecting them to an overnight blocking (of non-specific binding) at 4^{o}C in 3% BSA in PBS (pH 7.4) in a moist chamber with gentle shaking. On the next day, primary antibodies dissolved in 3% BSA in PBS (pH 7.4) were smeared onto the

slides and the slides were then incubated at room temperature, in a dark moist chamber for an hour. After removal of the primary antibody solution and after several washes in PBS (pH 7.4), secondary antibody dissolved in 3% BSA in PBS was smeared on to the slides and the slides were incubated in a dark moist chamber at room temperature for 2 hours. Once the secondary antibody was removed and the slides were washed thrice in PBS (pH 7.4), Hoechst 33258 (10ug/ ml) (PBS: Hoechst=1:50000) dissolved in PBS was added onto the slides to stain the parasite nuclei prior to covering with a cover slip and sealing the sides with nail varnish and microscopy. When this fixation technique was followed, the surface signal of infected and non-infected erythrocytes were found to be stronger than were found when paraformaldehyde supplemented with 0.0075% glutaraldehyde was used. However there was severe damage to internal structures. Hence it was used only for a few experiments to compare the signals found in different fixation techniques but was discontinued for regular experiments.

2.3.7.2.4 Image J/image processing and presentation

Image J, an open source Java-written program (http://rsbweb.nih.gov.ij/) was used to process all the images I took. The images photographed with the Axiovision microscope were imported to ImageJ64 and were converted to 8 bit greyscale images. Each image was subjected to background subtraction. Using the plug-in RGBmerge the channels were split between red, green and blue before individual images were overlaid to create a composite image. Images were converted to grey-scale using hyper stack function for better distinction between signal and background. In some situations images were taken at a higher exposure and were processed similarly thereafter. All the images were saved as TIF files. Images were imported to PowerPoint (Microsoft) from ImageJ program and were assembled.

2.3.7.3 Electron Microscopy

In order to analyse the ultrastructure of PVM and subsequently to understand the recruitment of erythrocyte membrane proteins onto the PVM, non-infected and *Plasmodium falciparum* and *Babesia divergens* infected erythrocytes were studied under EM. Parasites cultured, were adjusted as per required parasitemia. The cells were then fixed-permeabilized and sent to my collaboration partners in University of Oslo, Norway for sectioning and electron microscopy.

2.3.7.3.1 Preparation of cells for Electron microscopy

For the ultrastructure analysis, erythrocytes infected with *Plasmodium falciparum* and/or *Babesia divergens* were fixed by adding 2% glutaraldehyde in 200 mM HEPES in a ratio of 1:1 and were incubated at 37^{o}C for 5 minutes with gentle shaking. The contents were then centrifuged at 1600 x g for 2 minutes. The pellet was re-suspended in a solution containing 1% glutaraldehyde in 100 mM HEPES and incubated overnight at room temperature. On the next day the contents were centrifuged at 1600 x g for 2 minutes. The pellet was dissolved in 100 mM HEPES and was shipped to Oslo.

For Tokuyasu thawed cryo-sections, erythrocytes infected with *Plasmodium falciparum* and *Babesia divergens* were fixed by adding warm 8% paraformaldehyde solution dissolved in 200 mM HEPES in a ratio of 1:1(parasite culture : paraformaldehyde) and were incubated at 37^{o}C for 2 hours with gentle stirring. The contents were then centrifuged at 1600 x g for 2 minutes and the pellet was dissolved in 100mM HEPES and was shipped to Oslo for subsequent treatments.

3 Results

The majority of the information, available about erythrocyte invasion by Apicomplexan parasites, have been gathered from studies on different species of *Plasmodium* (Crabb et al., 1997, Tham et al., 2012, Cowman and Crabb, 2006, Crabb and Cooke, 2004). The parasite receptors and their specific roles in the active invasion had been rather well characterised. However the involvement and roles of different RBC membrane components during invasion and later in the formation of the PVM, has remained largely enigmatic. The rationale behind the inclusion or exclusion of specific erythrocyte membrane components in the PVM had also undergone major changes over time.

The initial hypothesis, as reviewed by Lingelbach and Joiner in 1998 (Lingelbach and Joiner, 1998) about inclusion of membrane lipids onto the PVM by the invading parasite, but selective exclusion of major erythrocyte membrane proteins, like Band 3, Glycophorin and Spectrin (Atkinson and Aikawa, 1990, Dluzewski et al., 1989, Ward et al., 1993) had also undergone major changes over later years. Several independent studies have shown that at least two multiple membrane spanning proteins namely Aquaporin-1 (Murphy et al., 2004)and Aquaporin-3 (Bietz et al., 2009) and several GPI anchored proteins like Flotillin-1 and -2 and CD59 are associated with the PVM in *Plasmodium* spp.(Murphy et al., 2004, Murphy et al., 2006). However major erythrocyte membrane proteins had reportedly been found absent from the PVM and the association of these proteins with the erythrocyte cytoskeleton had been reasoned to be behind this absence (Lauer et al., 2000, Murphy et al., 2004).

For a broader understanding of the components of PVM, with emphasis to the contributions of the parasite and of the host erythrocyte, I compared the components of PVM generated during the erythrocyte invasion by two Apicomplexan parasites; *Babesia divergens* and *Plasmodium falciparum*. Using a *Babesia divergens* strain, adapted to human erythrocytes allowed me to compare the cell biology of *P. falciparum* and *B. divergens* maintained in the same host cell and thereafter monitor the recruitment of RBCM proteins from the same donor cell by these two different parasites.

3.1 Synchronisation experiments for *Babesia divergens*

In *Babesia* spp. the PVM disintegrates soon after invasion, leaving the parasite free inside the erythrocyte (Rudzinska et al., 1976, Repnik et al., 2015). Unlike in *Plasmodium falciparum*, the growth of *Babesia divergens* had not been well synchronised and therefore the study of PVM, its formation and/or dissociation becomes all the more difficult.

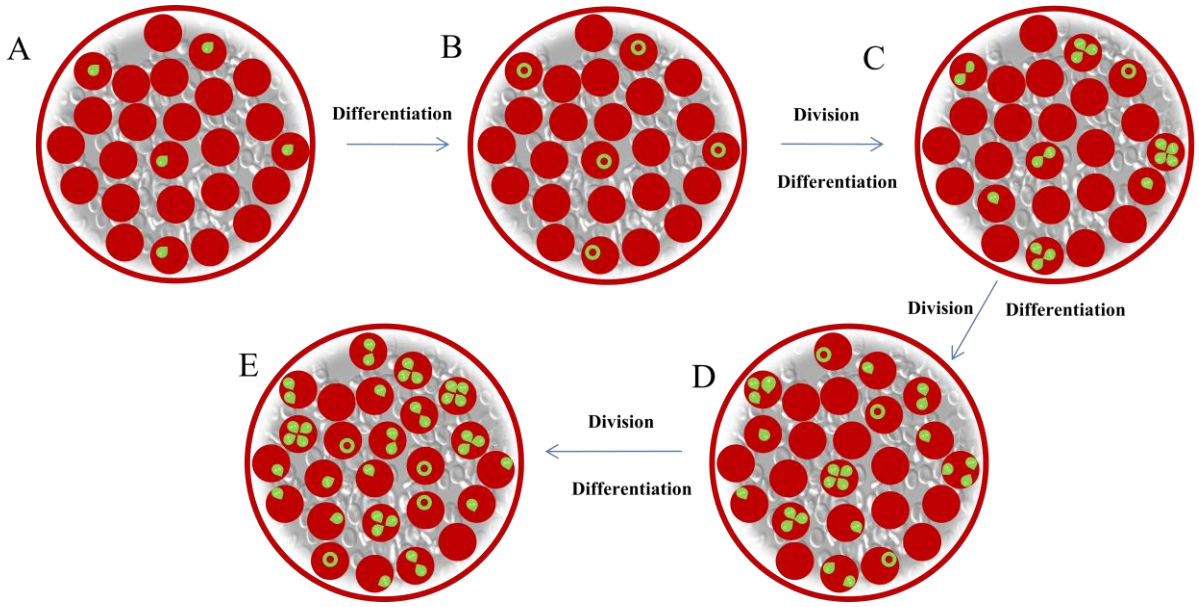


Figure 3.1: Development and asexual stages of *Babesia divergens*: a relative idea of parasitemia with 25 RBCs *in vitro*

A) Five piriform merozoite infect five RBCs, Parasitemia of (5/25)100=20%; the merozoites will differentiate to form differentiated merozoites (denoted by ring like structures for easier comprehension)

B) Five piriform merozoites inside the RBCs differentiate to form five differentiated merozoites; Parasitemia (5/25)100=20%. So far the development is moderately synchronous. However these differentiated merozoites lose the PVM and divide to become trophozoites (2-4) and this process takes different time in different parasites. Moreover multiple infections are common phenomena in *Babesia* spp. and this adds to the asynchrony in growth.

C) The differentiated merozoites lose the PVM and undergo nuclear division; giving rise to different numbers of trophozoites and the trophozoites grow and divide by budding like division forming 2 or 4 nucleated clusters of *Babesia* inside the RBC. However, multiple old infections and fresh infection running in parallel starts causing minor alterations in the parasitemia (8/25)100=32%.

D) The clusters of *Babesia* spp. dislodge and the piriform merozoites egress at different time points to invade fresh RBC and to carry on with the infection. The culture grows more and more asynchronous. Some erythrocytes have a few old merozoites from old piriform merozoite clusters left within, whereas some parasites break he clusters and egresses to invade new erythrocytes; *Babesia* can attain a moderately high parasitemia of about 50%-60% by this time of without much cell lysis or parasite death.

E) The culture grows up to a very high parasitemia of around 75%-80% with equally high asynchrony. The parasites inside can be in every possible stages of their differentiation and growth i.e. piriform merozoites in there Maltese cross like arrangement, merozoite clusters, trophozoites and differentiated merozoites alike.

In *Babesia divergens* the PVM is lost early during the infection, soon after invasion and prior to any nuclear division. Hence to understand the components of the PVM, its formation and disintegration, it is beneficial to have more of the early stages of the parasite. But unlike in *Plasmodium falciparum*, there are no apparent morphological demarcations to distinguish between the early and later single-nucleated stages of the *B. divergens*. The post nuclear division developmental stages of the parasite are however distinguishable owing to their unique shapes and organization inside the erythrocyte (Repnik et al., 2015, Rudzinska et al., 1976).

Cell biological and morphological alterations caused to the host erythrocyte, during the later stages of infection by the resident *P. falciparum* are routinely exploited to achieve synchronisation of such infected erythrocytes. Chemical treatments like Gelafundin (Pasvol et al., 1978) and Sorbitol (Lambros and Vanderberg, 1979) can achieve an enrichment of up to 40-50% of the later trophozoite and earlier ring stages respectively. Moreover by an unobtrusive physical method of applying high intensity magnetic field (Paul et al., 1981) around 80% enrichment of the trophozoite stages can be achieved. (Kindly refer to the Table 3.1-3.3 for detailed principle)

Similar morphological alterations are also reported in *B. divergens* infected erythrocytes (Hutchings et al., 2007, Alkhalil et al., 2007). If I could separate the later stages of *Babesia* spp. exploiting the morphological and cell biological alteration of the infected erythrocytes; it would indirectly help me enrich the earlier stages. With this assumption, I made several attempts to synchronise *Babesia divergens* infected erythrocytes. I followed the same techniques used in *Plasmodium falciparum*; but the synchronisation attempts failed on *B. divergens.*

The observations are tabulated in the next two pages.

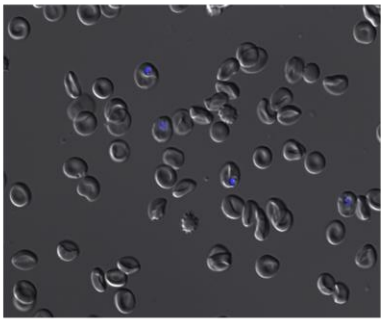
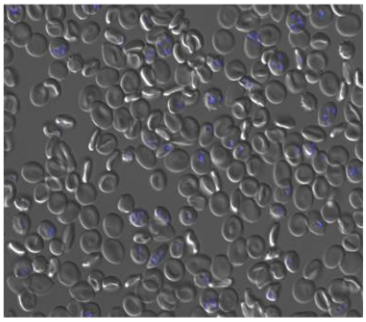
Treatment	*Plasmodium falciparum*	*Babesia divergens*
Gelafundin: Pasvol Wilson et al. 1978 In the later stages of the infection by *Plasmodium* spp., knob (Luse et al 1971) like structures form beneath the erythrocyte surface and this lead to an altered buoyancy of the erythrocytes. While treated with gelafundin in a falcon tube, these cells with higher buoyancy float on top and get separated from the other stages of infection, sedimenting at the bottom. This top layer is isolated and is found enriched with the trophozoites.	*Plasmodium falciparum* was found enriched with trophozoites after treatment with gelafundin.  **A**	Gelafundin treatment had no effect on *Babesia divergens infected* erythrocytes and synchronization could not be achieved.  **B**

Table 3.1: Effects of Gelafundin on *Plasmodium falciparum-and Babesia divergens*-iRBC: Treating with Gelafundin leads to enrichment of the trophozoite stages of the *Plasmodium* spp. whereas the ring stages are lost (A). After incubation with no-infected erythrocytes for an hour, the trophozoite stages are seen under microscope (A) On erythrocyte infected with *Babesia divergens*, there was no visible effect of Gelafundin (B), could be seen, the parasites were always a mixed state

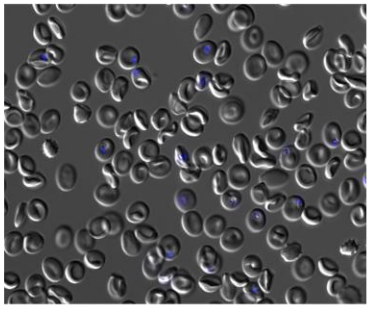
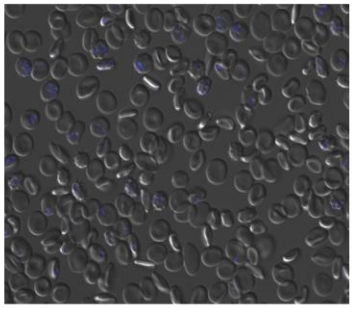
Treatment	*Plasmodium falciparum*	*Babesia divergens*
Sorbitol: Lambros and Vanderberg 1979 Sorbitol exploits the altered permeability of infected erythrocytes in the later stages of infection by the invading apicomplexan. Sorbitol enters the erythrocytes and causes an osmotic imbalance inside these cells ultimately leading to their destruction. Non-infected erythrocytes and erythrocytes containing the parasite in earlier stages can avoid such imbalance and survives in turn getting separated.	The culture of *Plasmodium falciparum* infected erythrocytes were found enriched with rings after these were treated with Sorbitol. Sorbitol does not affect non-infected cells and such cells remained intact after the treatment  **A**	Soribtol treatment *had no effect on Babesia divergens* infected erythrocytes and all the developmental stages were found in the culture before and after the treatment. Sorbitol had no effect on non-infected cells.  **B**

Table 3.2: Effects of Sorbitol on *Plasmodium falciparum-and Babesia divergens*-iRBC: Treating with Sorbitol causes osmotic lysis of the *Plasmodium falciparum*-iRBC containing old trophozoite stages, leaving the early ring stages intact (A). Non-infected erythrocytes are resistant to sorbitol treatment, and were found intact after the treatment. (A) After an hour of incubation with non-infected erythrocytes, these ring stages can be seen visibly enriched On erythrocyte infected with *Babesia divergens*, there was no visible effect of Sorbitol (B) neither any cell lysis was visible during treatment. The parasites were asynchronous before or after the treatments.

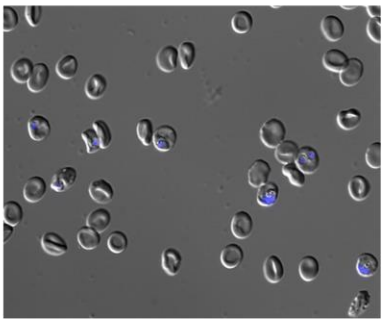
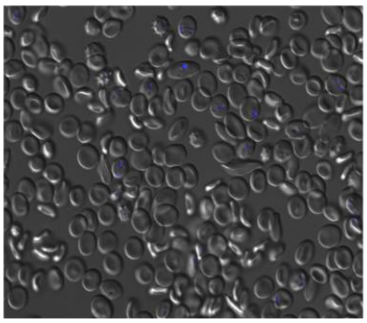
Treatment	*Plasmodium falciparum*	*Babesia divergens*
High intensity magnetic field (Paul, Roath et al. 1981) The nutrient source inside the erythrocyte is Haemoglobbin and the parasites feed on the same. It digests the haemoglobin to use the peptic digestive product and the metallic haem is not digested and remains residual of digestion. In later stages of the *Plasmodium falciparum* the ferromagnetic and/or diamagnetic Haem' accumulates inside the food vacuole in the form 'Haemozoin' Application of a high intensity magnetic field causes arrest of such cells in a flow column fitted in a magnetic field whereas early stages devoid of accumulated haemozoin can run through	The culture of *Plasmodium falciparum* infected erythrocytes were found enriched with trophozoite stages after ther were passed through the high intensity magnetic field.  **A**	No effect of the high intensity magnetic field based separation technique aas seen on *Babesia divergens* infected erythrocytes. The cells were in mixed stage of infection before and after the treatment  **B**

Table 3.3: Effect of a high intensity magnetic field on *Plasmodium falciparum*-iRBC and on *Babesia divergens*-iRBC.

Application of high intensity magnetic field arrests the iRBC containing the trophozoite stages and schizonts stages of the *Plasmodium falciparum* and these are distinguishable under microscope owing to their nuclear staining and on closer inspection by food vacuole(not distinguishable here). (A) On erythrocyte infected with *Babesia divergens*, there was no visible effect of high intensity magnetic field (B).There was neither an enrichment of any specific stages nor elimination of any other. The parasites were always a mixed state

3.1.1 Transient enrichment of *Babesia divergens*

Though the regular synchronisation methods failed on *Babesia* spp., I could achieve a transient enrichment of the differentiated merozoite stages, by allowing a controlled contact between non-infected RBC to a pellet of infected RBC with a very high parasitemia (80%-90%) before carefully isolating the top layer of the RBC-iRBC pellet and cell culture. The initial synchrony and the gradual loss of it are monitored over time under microscope by making a smear of infected erythrocytes, with or without nuclear staining.

This protocol was typically used for getting very early stages of invading merozoite and it allows infection of the non-infected erythrocytes by the parasites, when these are layered on top of an iRBC pellet. In the initial stages the infection, the rate of infection being low and moderately simultaneous, the invading merozoites differentiate and mature into trophozoites almost synchronously (Fig.3.2A-B). However these early trophozoites mature and divide to form mature trophozoites and piriform merozoite (beyond 8-10 hours). Ultimately giving rise to 2 or 4 progenies (Fig.3.2B-C). In *Babesia*, multiple infections are an often found phenomenon. Differentiation and development of these parasites, residing in the same erythrocyte takes different amount of time and these factors altogether lead to asynchrony in the culture(16 hours onwards) (Fig.3.2C-D).

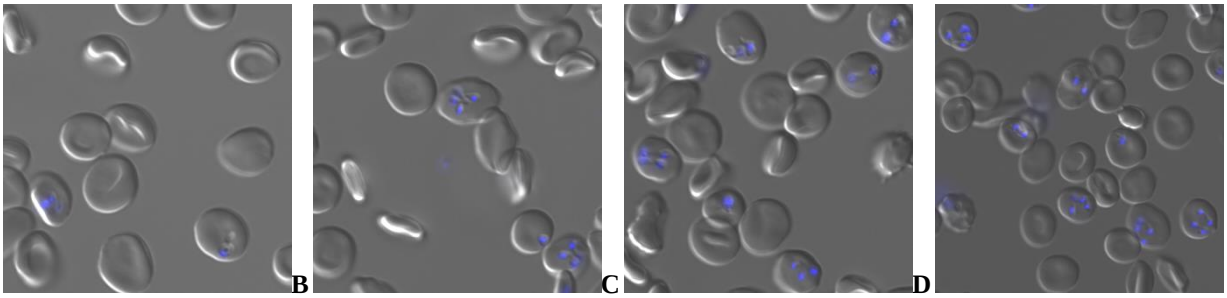


Figure 3.2: Transient enrichment of *Babesia divergens*: Monitored over 2-16 hours

Parasites were taken from culture, washed with PBS and were stained with Hoechst (1:50,000) in PBS prior to microscopy. Hoechst was used to identify the parasites nuclei and DIC was used to identify the erythrocytes. A) Initial stages of infection, very low rate of infection but the parasites are all in similar stage. B) The parasites divide and progress through their developmental cycle and the initial synchrony is partially lost. C and D) progression of infection leads to loss of the synchrony in culture.

The enrichment of the earlier stages of *B. divergens* was found to be less than 10%, as the rate of infection itself was low in this procedure (5% approx). But for some experiments when i needed early stages of infection I followed these procedures prior to preparing my samples.

A major reason behind attempting to synchronise the earlier stages of *B. divergens* was to study the formation and disintegration of the PVM in the early stages of infection. The invading merozoites of *Babesia* differentiate to give rise to the differentiated merozoites. But they do not undergo any nuclear division at this stage (Rudzinska et al., 1976, Repnik et al., 2015). The PVM is lost after this differentiation and nuclear division follows, giving rise to 2-4 trophozoite and piriform merozoite stages of the parasite. Erythrocyte membrane lipids are known to contribute towards the newly formed PVM. Therefore I labelled the erythrocyte membrane lipids by a non-transferable lipid analog conjugated with a dye molecule and allowed these labelled erythrocytes be invaded by *Babesia divergens*. The invasion and subsequent events were followed live, under microscope. This allowed me to see the formation of the PVM over a time scale of 1-10 seconds post invasion.

The erythrocytes were labelled with PKH26 dye following the manufacturer's guidelines. These labelled erythrocytes were then allowed to be invaded by *Babesia divergens* by incubating the PKH26 labelled non-infected erythrocytes with a *Babesia divergens* culture with a very high parasitemia of around 95% (based on regular haematocrit calculations). Half of the total samples were used for imaging live cells under microscope. Other half was fixed-permeabilized and prepared for IFA experiments. For live cell imaging, the exposure to laser was kept at the minimal level ≤95 milliseconds to avoid photo bleaching and also to avoid cell lysis.

After invasion the (T=0 second) the erythrocyte membrane seemed destabilized and the surface signal of the PKH26 seemed low. However a sack like protrusion was seen from the erythrocyte surface to the interior and connected to the erythrocyte by a stalk like connection.

The presence of Hoechst signal confirms the presence of a parasite associated with this extension and confirms this to be the developing PVM. The stalk like connection between the parasite and the erythrocyte membrane gradually shrinks (T=2-8 seconds). The parasite inside the sack rounds up and the erythrocyte surface signal seemingly increases, denoting membrane re-stabilization. Finally the stalk disappears (T=10 seconds) and the parasite is found surrounded in the pouch inside the erythrocyte.

However the erythrocytes started losing their structural integrity after a while under the experimental conditions and eventually lysed, so I was not able to monitor the lysis of PVM.

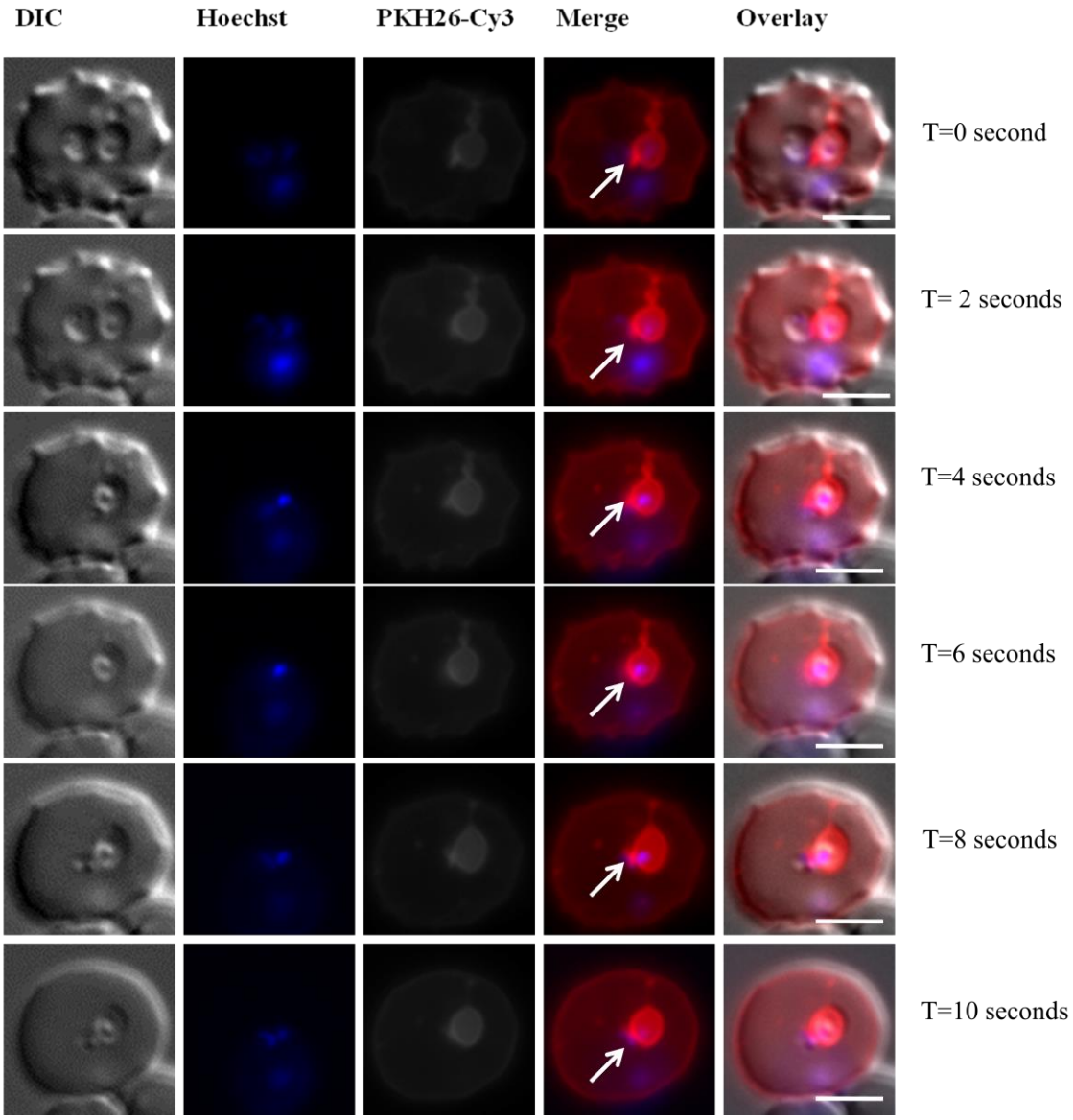


Figure 3.3: Formation of the PVM in the erythrocytes infected by *Babesia divergens*: Monitored by PKH26-Cy3 lipid staining over 10 seconds.

T=0 seconds: a stalk like projection connects the erythrocyte surface to the pouch like structure surrounding a parasite inside the erythrocyte. The parasite is distinguishable by the Hoechst signal corresponding to its nucleus. The surface signal is low at this point. T=2-8 seconds: The surface signal restores whereas the stalk like connection shrinks. In the end T=10 seconds: The parasite is found surrounded by a distinct pouch like structure inside the erythrocyte and the 'stalk' disappeared. Scale=3µm

3.2 Different fixation techniques and results:

I aimed at understanding the recruitment of erythrocyte membrane proteins to the newly formed PVM by comparing the invasion of erythrocytes by two related apicomplexan parasite *Babesia divergens* and *Plasmodium falciparum*. This study emphasizes highly on morphological aspects

of invasion induced compartmentation in the erythrocyte and is rich in immunofluorescence analysis. To be certain that my experimental results are not partial to any specific preparatory procedure of the sample materials, I did IFA with differentially fixed and permeabilised erythrocytes. The observations are summerised here

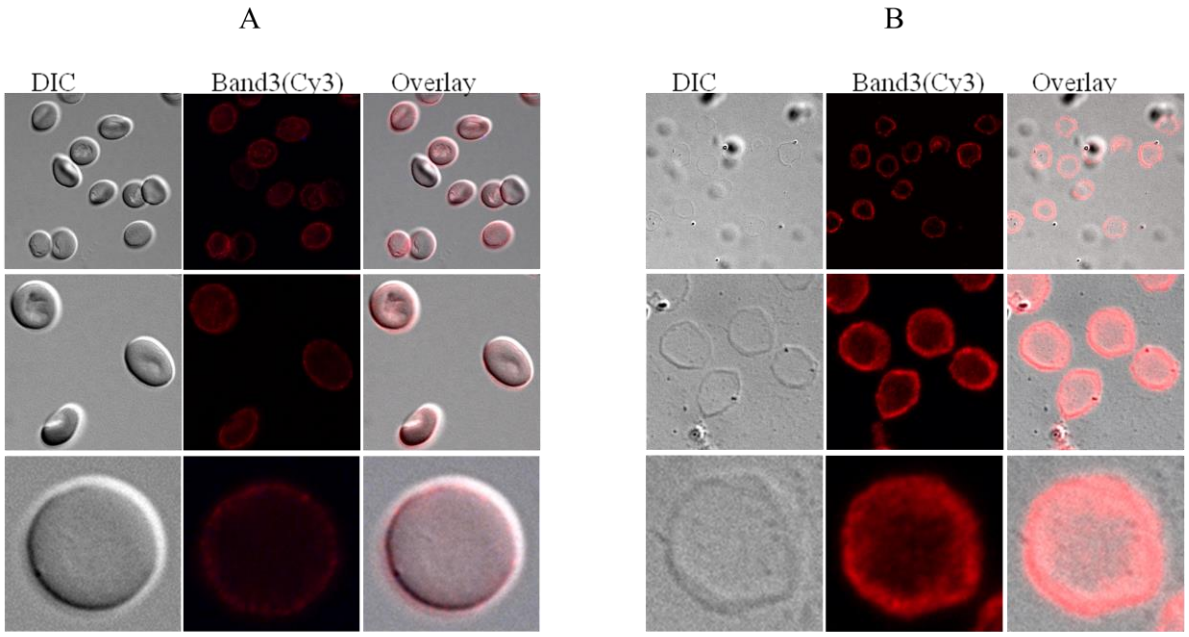


Figure 3.4: Comparison of Band 3 labelling patterns in un-infected RBC after fixing the cells following two different protocols. (PFD: Paraformaldehyde, GTD: Glutaraldehyde)

Fixation of the cell suspension with 4% PFD + 0/0075% GTD (A) gives a better structure preservation, but weaker labelling compared to the air-dried cell smear fixed with PFA alone (B). Cells were imaged at different magnifications. Rabbit monoclonal band 3antibody specific for the N-terminus of the molecule was used at 1:100, followed by Cy3-conjugated goat anti-rabbit secondary antibody.

(A) – erythrocytes in **a suspension** fixed with **4% PFD + 0.0075% GTD** in PBS or 30 min 37°C followed by membrane permeabilisation with 0.1% Triton X-100 in PBS for 15 min at room temperature - blocking of non specific binding with 3% BSA in PBS for 1 hour at room temperature - incubation with the primary antibody overnight at 4°C - incubation with the secondary antibody for 2 hours at room temperature

(B)- erythrocytes **smeared** on a glass slide, **air-dried** and fixed with **4% PFD** in PBS for 30 min 37 °C followed by- membrane permeabilisation with 0.1% Triton X-100 in PBS for 10 min at room temperature - blocking of non-specific binding with 3% BSA in PBS overnight at 4 °C - incubation with the primary antibody for 1 h at room temperature - incubation with the secondary antibody for 1 h at room temperature

Two of these fixation methods, using 4% paraformaldehyde (PFD) alone and in combination with 0.0075% glutaraldehyde (GTD) resulted better than other techniques; in terms of less cell lysis, higher structural preservation and proper immunolabelling. Hence I selected to go ahead with these two techniques for further experiments. However when tested on infected erythrocytes they yielded different results.The absence of glutaraldehyde during fixation resulted in stronger

on signals with the antibodies for their corresponding proteins on the erythrocyte surface but structures particularly the internal membranes were found to be poorly preserved

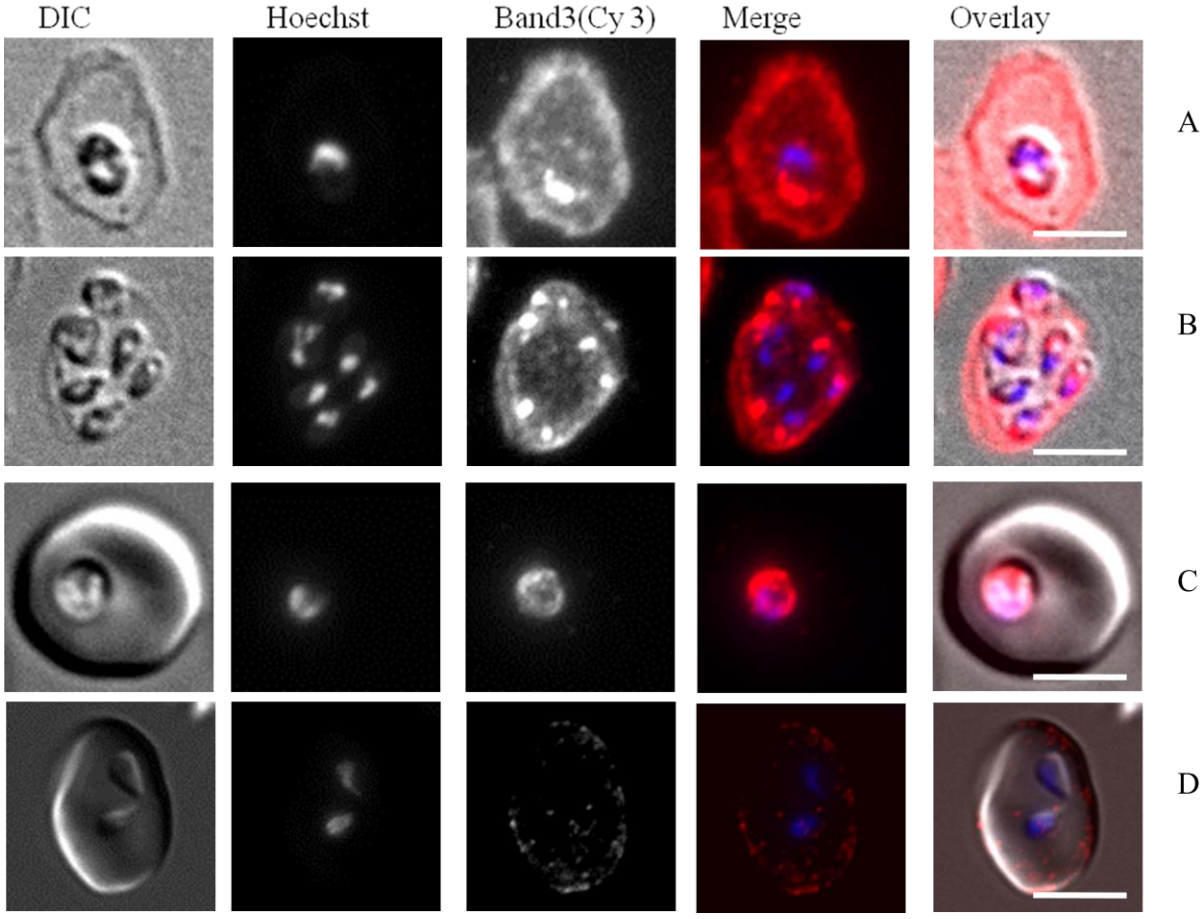


Figure 3.5: Comparison of the Band 3 labelling between differentially fixed *Babesia divergens*-infected erythrocytes.

A), B) Cells were fixed and labelled as was described for in Fig. 3.2B; C), D) Cells were fixed and labelled as was described for in Fig. 3.1 A; thereafter a rabbit monoclonal band 3 antibodies specific for the N-terminus of the molecule was used at 1:100, followed by Cy3-conjugated goat anti-rabbit secondary antibody. Parasites were identified with DIC and Hoechst nuclear staining In Fig.3.4 A, B, although the signal is strong, poor structural preservation makes the interpretation difficult and inconclusive. The internal signal in RBC infected with a single parasite may indicate PVM labelling (A). In the later stages, post nuclear division, no internal labelling corresponding to the Band 3 was noticed when the iRBC were fixed following this technique (B) However damage to the internal structures are clearly visible. Scale bar=3µm In Fig.3.4 C, D, surface signal was found to be low compared to what was found when treated with the other fixation method (Fig.3.4: A, B) but the erythrocyte structure and internal structures were visibly less damaged. The internal signal in RBC infected with a single parasite is indicative of the PVM labelling (C). In the later stages of development after nuclear division, no internal labelling corresponding to the Band 3 was noticed whatsoever (D).Scale bar=3µm

The focus of this study being elucidating the components of PVM and following it from its nascent stages through to its development both in *Plasmodium falciparum* and *Babesia divergens*

and/or its disintegration (*Babesia divergens*), any damage to internal structures would have been counterproductive. Thereafter all images were taken of cells fixed in the presence of glutaraldehyde.

3.3 Selected candidate proteins:

Proteins, typically containing glycosylphosphatidylinositol (GPI) anchors and associated with the detergent resistant microdomains of the plasma membrane are reportedly incorporated into the PVM of *Plasmodium falciparum* (Lauer et al., 2000, Murphy et al., 2004) infected erythrocytes. Over last decade, findings by us and by other groups have suggested, recruitment of at least two multiple spanning membrane proteins of RBC membrane Aquaporin 1 and 3 onto the PVM (Murphy et al., 2004, Bietz et al., 2009) whereas major membrane proteins such as Band 3, Spectrin and Glycophorin were found absent from the PVM (Atkinson and Aikawa, 1990, Dluzewski et al., 1992, Ward et al., 1993) and there had been many hypothesizes interpreting this absence (Lingelbach and Joiner, 1998, Fernandez-Pol et al., 2013). So far formation and the fate of the PVM in *Babesia divergens* had not been well described owing to the absence of suitable and specific marker proteins for the PVM, whereas much work had been studied in *Plasmodium falciparum*.

For this study I chose to compare the internalization pattern of a mixed set of proteins reportedly internalized and/or excluded from the PVM in *Plasmodium falciparum*. My reasoning was that during the active invasion, in analogy to *Plasmodium falciparum*, *Babesia divergens* might also recruit and exclude similar RBC membrane proteins. Thus these proteins could in turn be used as *bona fide* markers for the PVM. Selected protein candidates for this study were

- Flotillin-1(FLOT1_HUMAN_075955)
- Flotillin-2 (FLOT2_HUMAN_Q14254)
- CD59 (CD59_HUMAN_P13987)
- Aquaporin1 (AQP1_HUMAN_P29972)
- Aquaporin 3 (AQP3_HUMAN_
- Band 3 (B3AT_HUMAN_P02730)
- Spectrin α, β
- Glycophorin

I performed epifluorescence microscopy and biochemical analysis to analyze the recruitment of these candidate proteins onto the PVM developed during the invasion of human erythrocytes by *Babesia divergens* and *Plasmodium falciparum.*

I decided to use electron microscopy for elucidating the ultra-structure of *Babesia divergens* infected erythrocytes, their invasion induced compartmentation and also to confirm the recruitment of these proteins by immuno-EM.

In accordance to the description of Rudzinska (Rudzinska et al., 1976) in my thesis, stages of *Babesia divergens*, found surrounded by a vacuolar membrane (EM) or by components of RBCM (fluorescence microscopy) would be referred to as 'differentiated merozoites' whereas post nuclear division stages, not surrounded by any membrane components, would be referred to as either, 'trophozoites' or 'piriform merozoites (pear shaped).

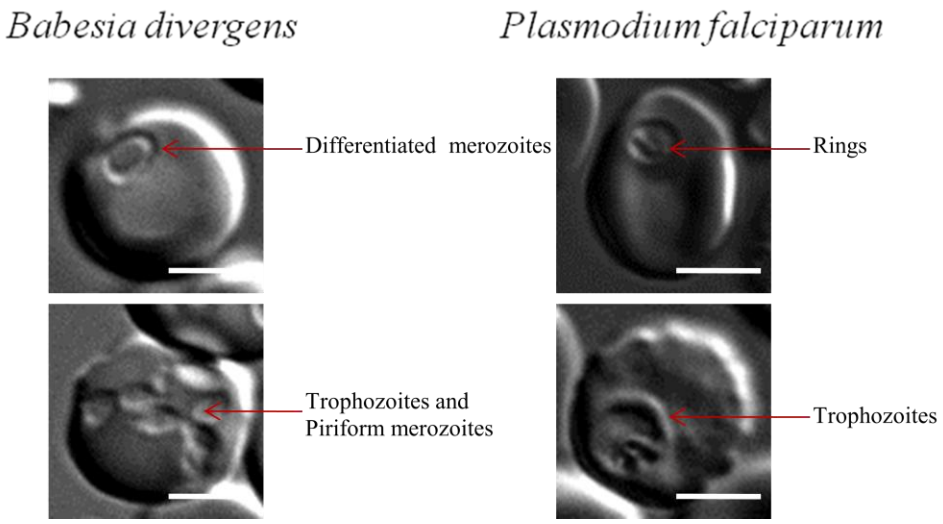


Figure 3.6: Different intra-erythrocytic stages of *Babesia divergens* and *Plasmodium falciparum*

In the left, panel on the top early single nucleated stages of the *Babesia divergens* is seen whereas the bottom panel shows post nuclear division trophozoites and piriform merozoites.

In the right, the panel on the top shows early ring stages of the invading plasmodium falciparum whereas the bottom panel shows the later trophozoite stages of the parasite.

In case of *Plasmodium falciparum* infected cells, the parasite without the typical food vacuole and loosely packed nuclear material, would be described to be 'rings' whereas the stages with defined, early or mature food vacuole and compact yet undivided nuclear material, would be categorized as 'trophozoites'.

All the Immunofluorescence experiments were done with *Plasmodium falciparum* infected and *Babesia divergens* infected erythrocytes in parallel, whereas non-infected erythrocytes were used as positive controls..

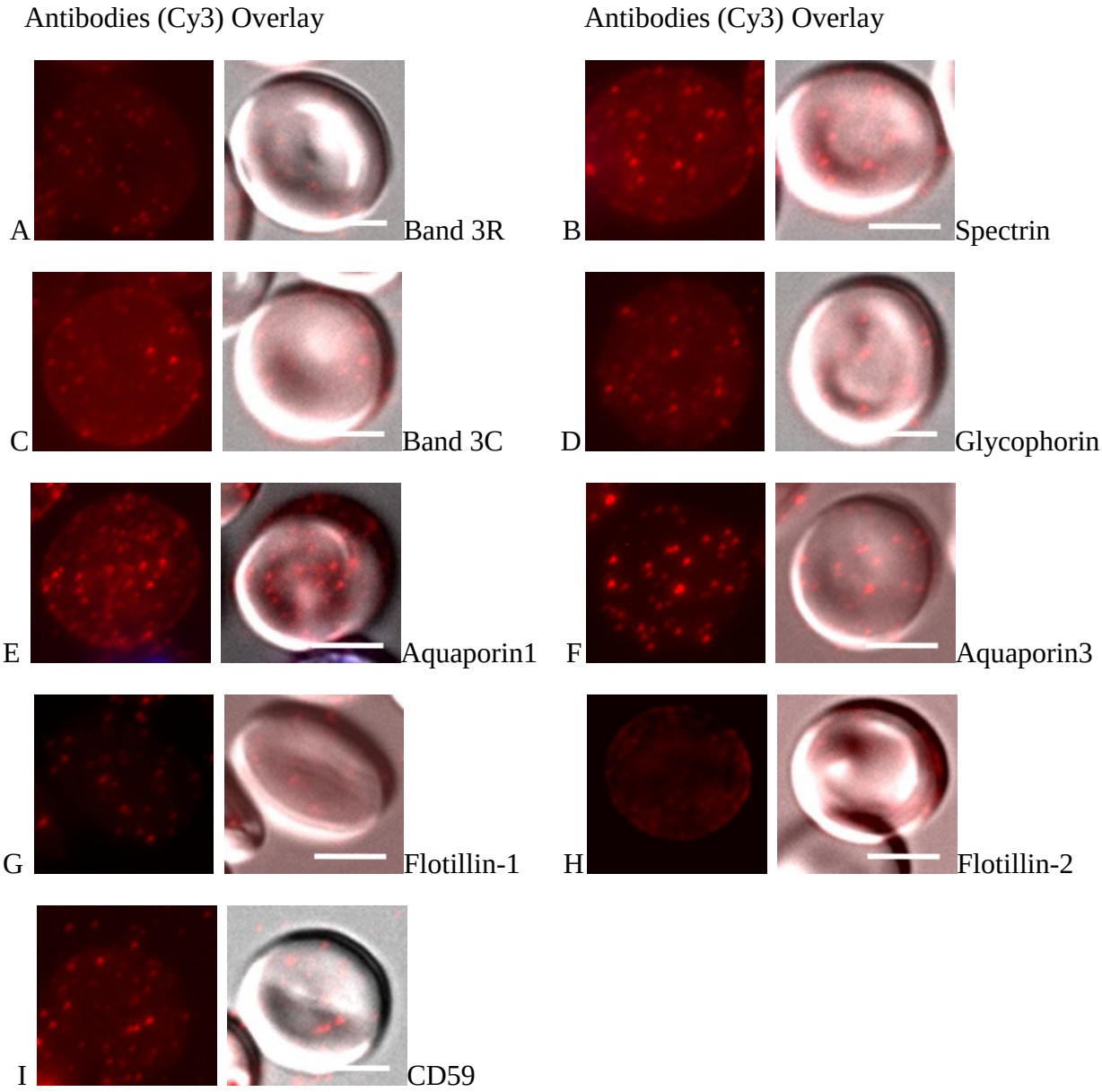


Figure 3.7: Immunolabelling of non-infected erythrocytes (positive controls) with antisera directed against erythrocyte membrane protein

A-I) Non-infected erythrocytes were used as positive controls for the immunostaining experiments. They were fixed-permeabilized-blocked against non-specific binding and thereafter immunolabelled with the antisera directed against the erythrocyte membrane proteins, in parallel to the infected erythrocytes (*Babesia divergens* and *Plasmodium falciparum*).

To avoid any confusion regarding difference in experimental procedures, treatment and about the imaging set ups, the results of the immunostaining for the erythrocyte membrane proteins on non-infected erythrocytes are being shown together. After incubation with secondary antisera-Cy3conjugate, the erythrocytes were imaged to demonstrate surface signal of the corresponding antibodies. The exposure was adjusted with secondary antibody staining (negative control) and the same exposure was used firstly to image the non-infected erythrocyte and later to image the parasite infected erythrocytes.

3.3.1 High abundance, membrane spanning and cytoskeleton associated protein of the of RBC membrane, Band 3 is found internalized and localized onto the PVM of cells infected with *Babesia divergens*

Over the last decade, the components of the PV and PVM had been under much scrutiny and the constituents had mostly been discussed about with the *Plasmodium falciparum* in focus. A major RBCM associated and cytoskeleton anchoring protein Band 3 had always been reported, non-incorporated onto the PVM (Lauer et al., 2000, Bietz et al., 2009, Murphy et al., 2004, Murphy et al., 2006, Ward et al., 1993). This observation had been reasoned suitably all along and the association of Band 3 with the erythrocyte cytoskeleton, had been attributed towards its absence from the newly formed PVM (Lingelbach and Joiner, 1998, Fernandez-Pol et al., 2013).

To understand the fate of Band 3 in erythrocytes infected with *Babesia divergens*, I performed IFA on *Babesia divergens*-iRBC (with the parasites in their mixed stage of development). I used a commercial rabbit monoclonal anti Band 3 antibody (Band 3R) (Fig.3.8A-B) that has an epitope, present at the N-terminal region of the Band 3 peptide. After determining its specificity for the Band 3 protein (a specific band around 100kDa) by immuno-blotting (Fig.3.8C) I continued on IFA.

In a subset of such infected erythrocytes, where the parasite was still it its differentiated merozoites stage, I found signal corresponding to Band 3R (Fig 3.8A) overlapping the nuclear signal (at this resolution). However neither signal corresponding to the PVM nor any significant internal labelling were found in the erythrocytes where the parasite had already undergone nuclear division and was found present in their trophozoite or piriform merozoite stages, arranged typically as cluster or Maltese cross (Fig 3.8B). In many cases, cells were found invaded by multiple parasites but the immunofluorescence corresponding to the Band 3R was

found only in a fraction of the undivided parasites, whereas signal was completely lost from further differentiated stages. If more than one of the invading parasites were found to be in the single nucleated stages, suggesting rather shortly interspaced invasion events, the fluorescent signal associated with Band 3R was more often found in the one, noticeably more proximal to the erythrocyte plasma membrane (Fig 3.8A).

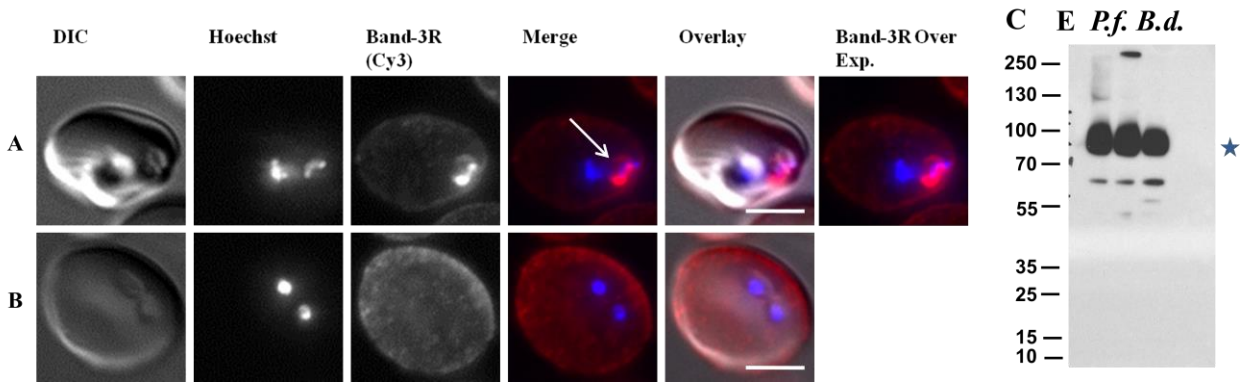


Figure 3.8: Whole mount IF labelling of Band 3 in erythrocytes infected with *Babesia divergens*

A-B: IF labelling of erythrocytes with rabbit polyclonal antibodies specific for the N-terminal region of Band 3 (Band 3R) (A, B) Parasites were identified with Hoechst nuclear staining and with Differential Interference Contrast (DIC) Channel. A fraction of iRBC infected with *Babesia divergens* was found having internal signal corresponding to Band 3R (A). iRBC containing parasites that had already undergone nuclear division did not show any specific internal labelling for either of the antibodies(B).Cells containing multiple nuclei were deliberately overexposed to exclude internal labelling and the over exposed images of the cells containing single nuclei are depicted on far right. White arrows indicate the PVM. Scale bar=3µm.

C: Immuno-blot analysis of whole cell lysate of erythrocytes, non-infected and infected with *Plasmodium falciparum* and *Babesia divergens*, $4*10^7$ parasite equivalent iRBC was subjected to hypotonic lysis and the membrane fractions were run on 10%SDS-PAGE gel, followed by semidry transfer and thereafter duly probed with the same polyclonal rabbit polyclonal Band 3R (E) The asterisk represents the band at 100kDa corresponding to the Band 3 polypeptide. At an exposure time of 10seconds, for the Band 3R (C) the signal appeared strong, a degradation product was detected in all of the lanes at around 60kDa and for the lane of *P.falciparum* iRBC another added band was detected at 260kDa.

In order to affirm the internalization of Band 3 and to conclude the signals from Band 3R are not any invasion related artifact, *i.e.* not a fragment of the large Band 3 peptide, I decided to repeat my experiments with another antibody directed against Band 3.

The commercial antibody (Band 3R) used was specific for an epitope at the N-terminus of the peptide, I decided upon an antibody with an epitope present around the C-terminus (Band 3C) (Fig.3.9A-B) of the peptide after determining its specificity by immuno-blotting (Fig.3.9C) This too yielded a 100kDa band (Fig.3.9C) but it was noticeable that in a same exposure time (10 seconds) the signal corresponding to Band 3R (Fig.3.8C) was stronger than the Band 3C

(Fig.3.9C). Moreover the antibody against Band 3R seemed to be detecting a degradation product of the Band 3 peptide at around 60kDa (Fig.3.8C).

IFA was carried out with fixed and permeabilized erythrocytes infected with *Babesia divergens* (in mixed stage of development).With this antibody (Band 3C), in a subset of infected erythrocytes, where the parasite was undivided and was in its differentiated merozoite stage, I found signal corresponding to Band 3, overlapping the nuclear signal (Fig.3.9A) (at this resolution). The erythrocytes those were invaded by multiples of *Babesia divergens*, the immunofluorescence signal corresponding to Band 3C was found only in the ones where the parasite had not undergone any division and was in the single nucleated stage. Signal was completely lost from the trophozoite and piriform merozoite stages (Fig.3.9B).

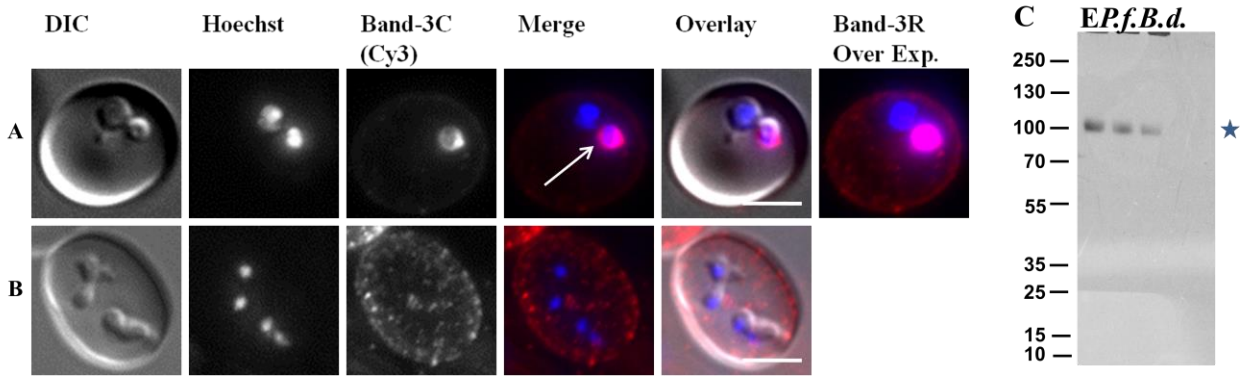


Figure 3.9: Whole mount IF labelling of Band 3 in erythrocytes infected with *Babesia divergens*

A-B: IF labelling of erythrocytes with rabbit polyclonal antibodies specific for the C-terminus (Band 3C) (A, B) Parasites were identified with Hoechst nuclear staining and with Differential Interference Contrast (DIC) Channel. A fraction of iRBC infected with *Babesia divergens* was found having internal signal corresponding to Band 3C, (A) specific for C terminal. iRBC containing parasites that had already undergone nuclear division did not show any specific internal labelling for either of the antibodies(B).Cells containing multiple nuclei were deliberately overexposed to exclude internal labelling and the over exposed images of the cells containing single nuclei are depicted on far right. White arrows indicate the PVM Scale Bar=3µm

C: Immuno-blot analysis of whole cell lysate of erythrocytes, non-infected and infected with *Plasmodium falciparum* and *Babesia divergens*, $4*10^7$ parasite equivalent iRBC was subjected to hypotonic lysis and the membrane fractions were run on 10%SDS-PAGE gel, followed by semidry transfer and thereafter duly probed with antibody against Band 3C (F) The asterisk represents the band at 100kDa corresponding to the Band 3 polypeptide.

In the erythrocytes where multiple of single nucleated Babesia divergens were found, more often than not the signal for Band 3C was found in the ones located noticeably proximally to the erythrocyte plasma membrane (Fig.3.9A). This observation also supports the fact that the PVM in *Babesia divergens* is lost very soon after invasion. In totality, neither PVM specific signal nor internal labelling could be found associated with the erythrocytes where the invading parasite,

had had undergone nuclear division and was found arranged in their 2 or 4 nucleated clusters or Maltese cross like arrangements (Fig.3.9B).

While *Babesia divergens* infected RBC were sent for EM to analyze the presence of internalized Band 3(Band 3C and Band 3R), the observations supported my findings and confirmed the presence of signals corresponding to Band 3 in a small subset of differentiated merozoite stages of *Babesia divergens*. EM also confirmed complete absence of such specific signal in any of the later differentiated 2 or 4 nuclear stages of *Babesia divergens* (Repnik et al., 2015).

3.3.2 Blocking the anti Band 3 antibodies with a synthetic peptide, followed by immunofluorescence and immuno-blotting analysis with the blocked –antibody, confirmed the internalization of Band 3 in erythrocytes infected with *Babesia divergens*

My observations on the internalization of Band 3 in *Babesia divergens* were against both the previous findings and the hypothesis in practice about the exclusion of this high abundance protein from PVM, owing to its close association with underlying cytoskeleton. Hence I chose to confirm my observations further and designed a control experiment to test the specificity of the antibodies used for Band 3 immuno-detection. I chose to block the antibodies and thereafter use them for immunodetection (immunofluorescence and immuno-blot) to check their specificity.

Immunolabelling (Fig.3.10A-H) was carried out on fixed and permeabilized infected erythrocytes (Fig.3.10, A-D and E-H) and with cell lysate of such infected erythrocytes (Fig. 3.10, I and J) with the antibodies against Band 3 i.e. Band 3R (Fig. 3.10A-D) and Band 3C (Fig. 3.8F-H) .Prior to immunolabelling the antibodies were treated with and without a prior incubation step with the synthetic peptide directed against the N-terminal region of Band 3 to block its epitope recognition (BP). Non-infected erythrocytes were used as control.

To block the immunoglobulin molecules present in a particular dilution (1/100 had given most optimal result in IFA so far) of the anti Band 3 antibody, amount of the synthetic peptide directed against the Band 3 commercial antibody (BP) required; was calculated from the manufacturer's guideline.

The antibodies (Band 3R and Band 3C) were thereafter incubated with the blocking peptide for 1 hour at room temperature. Then, the antibody-blocking peptide solutions were used in parallel for IFA and immuno-blotting, using the same batch of non-infected and *Babesia divergens*

infected erythrocytes. The same dilution of the antibodies without any blocking peptide was used as positive control.

In IFA (Fig. 3.10A-B), when the commercial rabbit monoclonal antibody against Band 3 (Band 3R) was used without any blocking peptide; I could find signals corresponding to the PVM in the differentiated merozoite stages of the parasite (Fig. 3.10A). However, I could not find any signal corresponding to the PVM, when the parasite had had undergone differentiation (Fig.3.10B). When IFA was done on cells from the same batch, with the same antibody (Band 3R) after suitably blocking with the synthetic peptide (BP) (Fig.3.10C-D) both internal and surface signal were completely lost.

Parallaly another set of IFA (Fig.3.10E-H) was carried out with *Babesia divergens* infected cells from the same batch, using the antibody against the C-terminal of Band 3 (Band 3C), with or without blocking it with the synthetic peptide.

Under microscope, signals corresponding to the Band 3C could be found in the differentiated merozoite stages of the parasite and (Fig.3.10E). However the internal signals were lost from the trophozoites or piriform merozoite stages of parasite.(Fig.3.10F).The internal and surface signals were retained irrespective of incubating the Band 3C antibody with the BP (Fig. 3.10G-H).

In the western blot analysis, with the lysate from the infected and non-infected erythrocytes of the same batch (Fig.3.10, I and J), the specificity of the synthetic peptide used for blocking (BP) the antibody Band 3R (Fig. 3.10I) (against which it was actually manufactured) and insensitivity for the other (Band 3C) (Fig. 3.10 J) was confirmed.

The specific band representing Band 3 on blot (around 100kDa) which both the antibodies recognized, was not recognized by the Band 3R after it was incubated with the BP (Fig.3.10I);however the band was still recognized by the Band 3C antibody even after incubation with the BP (Fig.3.10J).

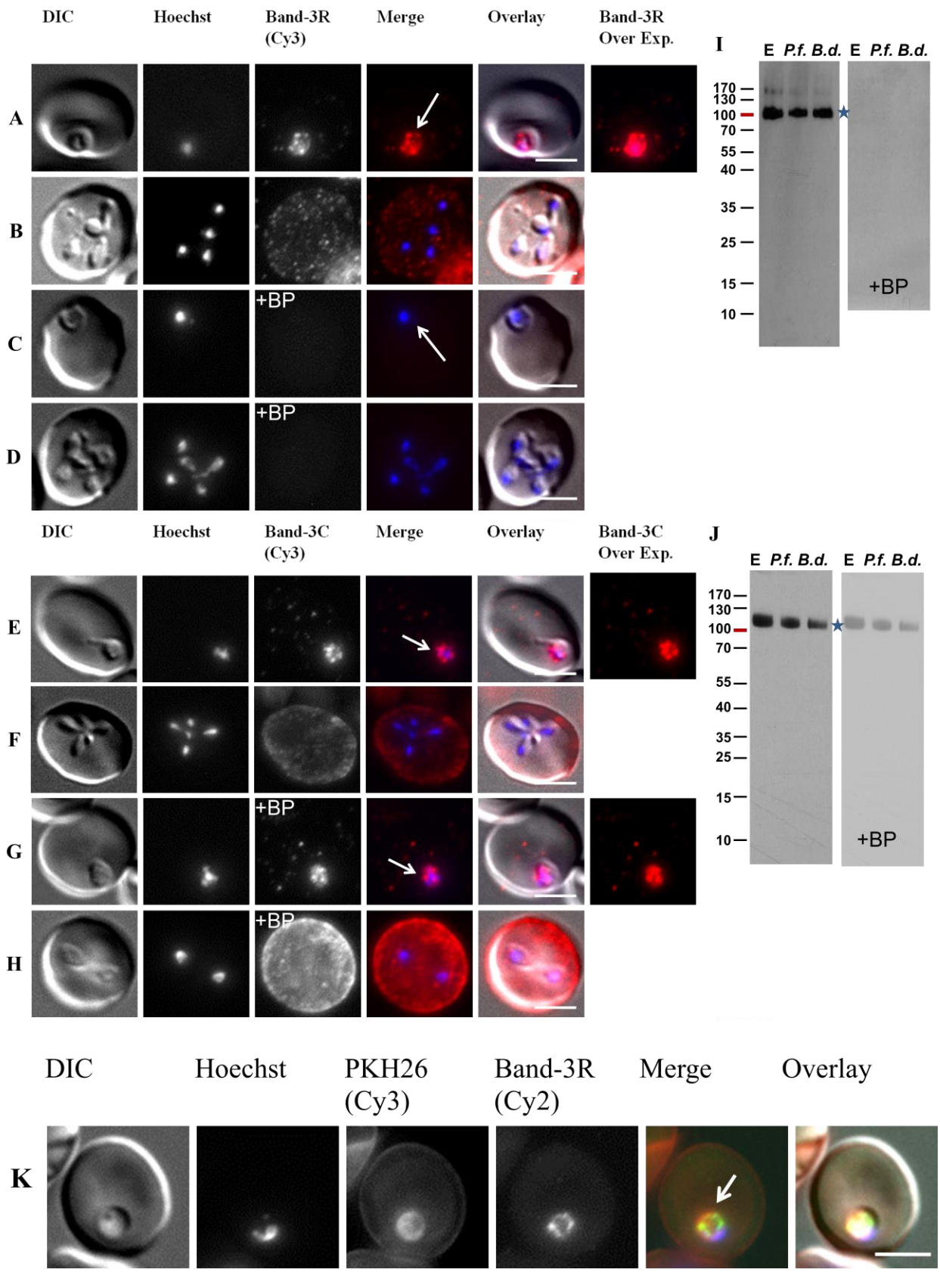

DIC
Hoechst
Band-3R (Cy3)
Merge
Overlay
Band-3R Over Exp.
A
B
C
D
+BP
+BP
I
E P.f. B.d. E P.f. B.d.
170
130
100
70
55
40
35
25
15
10
+BP
DIC
Hoechst
Band-3C (Cy3)
Merge
Overlay
Band-3C Over Exp.
E
F
G
H
+BP
+BP
J
E P.f. B.d. E P.f. B.d.
170
130
100
70
55
40
35
25
15
10
+BP
DIC
Hoechst
PKH26 (Cy3)
Band-3R (Cy2)
Merge
Overlay
K

Figure 3.10.Whole mount IF labelling of Band 3 in erythrocytes infected with *Babesia divergens* in presence or absence of a synthetic peptide targeted to block the antisera Band 3R

A-H: IF labelling with rabbit polyclonal antibodies specific for the N-terminal region of Band 3 (Band 3R) (A-D) and C-terminal (Band 3C) (E-H) in the absence (A, B, E, F) or presence (C, D, G, H) of a blocking peptide (BP) derived for against the N-terminal region of the Band 3 peptide. Scale Bar=3µm

Parasites were identified with Hoechst nuclear staining and with Differential Interference Contrast (DIC) channel. A fraction of iRBC infected with *Babesia divergens* was found having internal signal corresponding to Band 3R (A).specific for N-terminal and Band 3C, (E) specific for C terminal. IRBC containing parasites that had already undergone nuclear division did not show any specific internal labelling for either of the antibodies (B, F).

In the presence of a specific blocking peptide against the N-terminus of the Band 3 peptide, signal was lost for the antibody Band 3R (C, D) but was retained for the antibody Band 3C (G, H).Cells containing multiple nuclei were deliberately overexposed to exclude internal labelling and the over exposed images of the cells containing single nuclei are depicted on far right. White arrows indicate the PVM

I, J: Immuno-blot analysis of whole cell lysate of erythrocytes non-infected and infected with *Plasmodium falciparum* and *Babesia divergens.* $4*10^7$ parasite equivalent RBC and iRBC was suitably prepared and the membrane fractions were run on 10% SDS-PAGE gel, followed by semidry transfer and thereafter duly probed with the same rabbit polyclonal Band 3R (I) or Band 3C (J) antibodies; in the absence and presence of the blocking peptide against the N-terminal region of the Band 3 peptide. The asterisk represents the band at 100kDa corresponding to the Band 3 polypeptide.

K: Co-localization of the internalized Band 3 (Cy2) signal with internalized PKH26 (Cy3) signal, these two signals are in juxtaposition (representing the PVM of the single undivided parasite) to the Hoechst signal (representing the single undivided parasite). Scale Bar=3µm

After being confirmed of its internalization, I chose to investigate if the Band 3 co-localizes with the internalized membrane lipids of the nascent PVM (Fig.3.10K), membrane lipids of the non-infected erythrocytes were labelled using a commercial membrane lipid staining kit followed by invasion assay and microscopy. The lipophilic dye PKH26 present in this kit is believed to be non-transferable from the labelled cell's surface to that of unlabelled membranes during any shorter interaction (Ward et al., 1993).The cell labelling is carried out using a salt or solvent or buffer free aqueous Diluent, named Diluent C that has an osmoregulant added to it and is isotonic for mammalian cells at ~300 mosmol/L (Manufactures data).

The labelled cells were added to cultures of *Babesia divergens* and *Plasmodium falciparum* infected RBC and were maintained in culture to allow invasion to commence. The cells were then harvested and were duly fixed, permeabilized and immunolabelled with Band 3 specific antibody microscopy and study any co-localization of the Band 3 protein with any internalized labelled lipids.

For a subset of erythrocytes infected by *Babesia divergens* with the parasites in their uni-nucleated stages, PKH26 signal corresponding to the PVM was found present, surrounding a Hoechst signal corresponding the nucleus (at this resolution), however in cells of same culture,

that contained divided parasites or parasites in their piriform (pear shaped) stages as indicated by the Hoechst signals, did not have any internal signal corresponding to PKH26 whatsoever. In a subset of *Babesia divergens* infected erythrocytes with the PKH26 labelling the PVM, signals corresponding to Band 3R was found co-localizing with the PKH26 stains at the periphery of the single celled parasite indicating the co-localization of the protein and lipid stains onto the PVM (Fig.3.10K).

While the *Babesia divergens* infected RBC were sent for EM to analyze the presence of internalized Band 3, their observations supported my findings and confirmed the presence of signals corresponding to Band 3(Band 3C and Band 3R) in a small subset of differentiated merozoite stages of *Babesia divergens*. The signal was eventually lost from the differentiated stages of the parasite. EM also confirmed complete absence of such specific signal in any of the later 2 or 4 nuclear stages of *Babesia divergens* (Repnik et al., 2015).

3.3.3 Erythrocyte cytoskeletal protein, Spectrin was also found localized onto the PVM of *Babesia divergens* infected cells

On finding Band 3 to be recruited onto the PVM of *Babesia divergens* infected erythrocytes, I looked into the biological details of Band 3 and found that apart from being a very high copy number, membrane spanning protein of the erythrocyte, Band 3 is also very closely associated with the membrane cytoskeleton (Agre et al., 1988) and this association had been argued to be the reason behind its exclusion from the newly forming PVM during cell invasion by *Plasmodium falciparum* (Lauer et al., 2000, Murphy et al., 2004).Therefore I chose to next investigate, the localization of the major cytoskeletal protein Spectrin in cells infected with *Babesia divergens*. In erythrocytes, Spectrin is reportedly present lining the cytosolic side of the RBCM (Boguslawska et al., 2014). A commercial antibody against Human Spectrin (α and β) was used at a dilution of 1:100 for these IFA (Fig3.11A- B) after determining its specificity in immuno-blot analysis (Fig.3.11C)

In western blot analysis the antibody yielded specific bands representing Spectrin (β-chain at 220kDa and α-chain at 240kDa) but when exposed for longer time (1 minutes, compared to 10 seconds of previous exposure time) it yielded several other bands as well, which could be corresponding to the different heteromeric or homomeric units (Fig.3.11C). However when used

to label erythrocytes infected with *Babesia divergens* (mixed culture) the specific labelling was much stronger than any background labelling (Fig.3.11A- B).

In a subset of erythrocytes infected with *Babesia divergens* with the parasite yet to undergo division (indicated by a single Hoechst signal) (Fig.3.11A) an internal signal corresponding to Spectrin was found localized surrounding (at this resolution) the single nuclear signal of Hoechst. Like our observations for Band 3, the signal for Spectrin and the corresponding parasite was located much closer to the RBCM, indicating a relatively recent invasion event. No specific internal labelling was observed in cells where the parasite had already undergone division and had given rise to 2 or more parasite (Fig.3.11B).

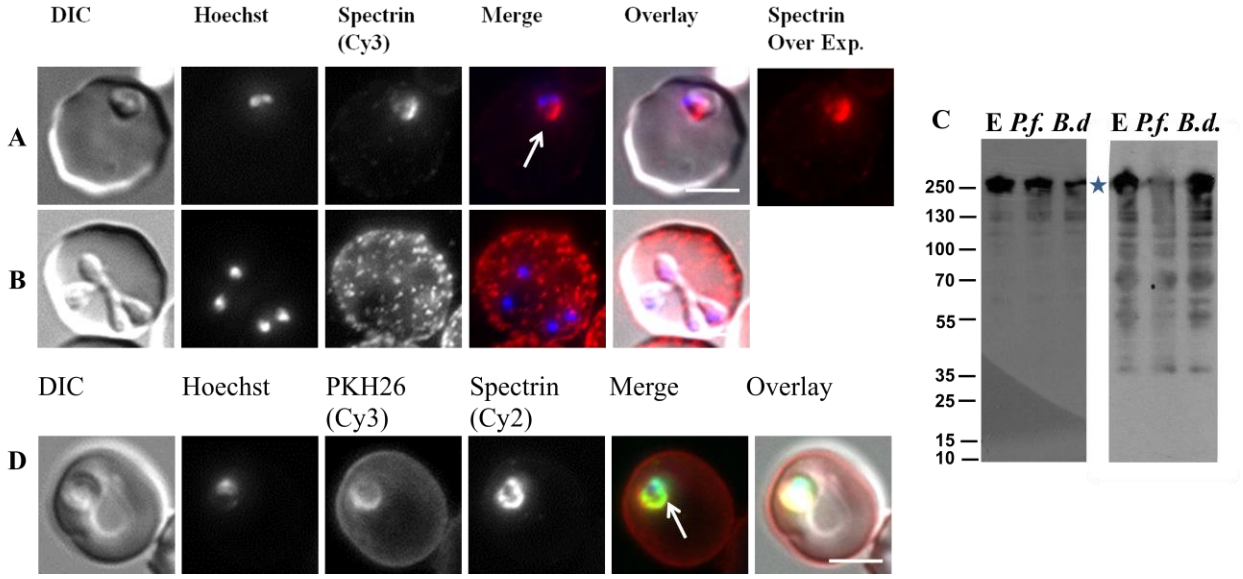


Figure 3.11: Internalization of Spectrin onto the PVM of erythrocytes infected with *Babesia divergens*

A, B: Whole mount IF labelling, Parasites were identified with Hoechst nuclear staining and with Differential Interference Contrast (DIC) .Internal signal corresponding to the Spectrin was observed in a small fraction of RBC containing the parasites prior to their nuclear division (A) but not in the cells already undergone the division (B) An overexposed image of a RBC containing single parasite is shown on far right. White arrows denote the PVM. Scale Bar=3µm

C: Immuno-blot analysis of whole cell lysate of erythrocytes, non-infected and infected with *Plasmodium falciparum* and *Babesia divergens*, $4*10^7$ parasite equivalent RBC and iRBC was suitably prepared and the membrane fractions were run on 10% SDS-PAGE gel, followed by semidry transfer and thereafter duly probed with the probed with anti-spectrin antibody, the panel on the right shows higher exposure time and the asterisk sign demarks the bands corresponding to alpha and beta chains of spectrin at 240kDa and 220kDa respectively. Many degradation products were detected at a higher exposure (60 seconds, compared to 10seconds of lower exposure)

D: Co-localization of internalized Spectrin (Cy2) and PKH-26 (Cy3) in the parasite periphery. These two signals are in juxtaposition, representing the protein and lipid entities of the PVM in the single undivided parasite, represented by to the Hoechst signal. Scale Bar=3µm

To identify any possible co-localization of the Spectrin with the membrane lipids onto the nascent PVM (Fig.3.11D) of cells infected by *Babesia divergens*, an experiment similar in line to that done for Band 3, *was* designed. Membrane lipids of non-infected erythrocytes were labelled using the commercial PKH26 membrane lipid staining kit followed by invasion assay, harvest, and immunolabelling with anti Spectrin antibody followed by microscopy

As was observed for Band 3, for a set of erythrocytes infected by *Babesia divergens* where the parasites were in their differentiated merozoite stages, PKH26 signal corresponding to the PVM was found surrounding the Hoechst signal (Fig.3.11D) whereas in cells that contained divided parasites or parasites in their piriform (pear shaped) stages no internal signal corresponding to PKH26 whatsoever to be found. Furthermore in a subset of such *Babesia divergens* infected erythrocytes where the PKH26 labelled the PVM, signals corresponding to Spectrin was found co-localizing with the PKH26 stains around the periphery of these single celled parasite indicating the co-localization of the protein and lipid stains on the PVM (Fig.3.11D).

While erythrocytes infected with *Babesia divergens* were studied under EM, the observations confirmed my findings and affirmed the presence of signals corresponding to Spectrin in the early uni-nucleated stages of *Babesia divergens* and absence of associated signal in the later stages with divided nuclei. On immuno-gold labelling of high pressure frozen cells (performed in Oslo) and EM analysis, the signals corresponding to Spectrin was found at the parasite periphery delineating the parasitophorous vacuole. However such signals were completely lost when the parasite had undergone division and was found to be present in its trophozoite and piriform merozoite stages (Repnik et al., 2015).

3.3.4 Though not found on standard IFA but under EM Glycophorin-A was found to be internalized in cells infected by *Babesia divergens*.

A high copy number RBCM protein Glycophorin had always been reported not internalized in the PVM of erythrocytes infected by *Plasmodium falciparum* (Atkinson and Aikawa, 1990, Dluzewski et al., 1989, Ward et al., 1993) and this finding which had been interpreted as the result of a selective exclusion of host cell membrane proteins by these parasites ((Lingelbach and Joiner, 1998).

However Glycophorins too, like Band 3 have been shown to interact with the receptors of *Plasmodium falciparum* and *Babesia divergens* during the cellular invasion of these parasites

(Pasvol, 2003, Pasvol et al., 1982a, Pasvol et al., 1982b, Lobo, 2005, Cursino-Santos et al., 2014b);. Hence I chose to investigate next, the incorporation of Glycophorin-A in the PVM of *Babesia divergens*.

I used a commercial mouse monoclonal antibody, specific towards the extracellular part of the Glycophorin-A molecule and in western blot analysis the antibody appeared to be specific for the Glycophorin-A protein (Fig.3.12 C).

On performing IFA (Fig.3.12, A-B)on cells infected by *Babesia divergens* in their mixed stages of development, I failed to identify any specific labelling of Glycophorin-A corresponding to any undivided nuclear signal from Hoechst (Fig.3.12, A- B) or to any of the later stages of the parasite, in spite of a rather strong surface signal.

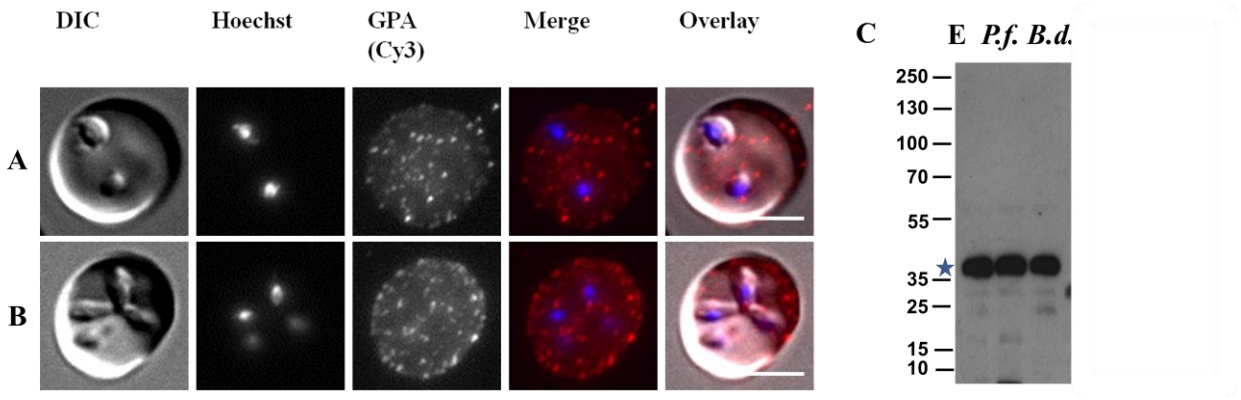


Figure 3.12: Glycophorin-A (GPA) labelling of erythrocytes infected with *Babesia divergens*

A, B: Whole mount IF labelling of erythrocytes infected with *Babesia divergens*, before and after nuclear division... Parasites were identified with Hoechst nuclear staining and with Differential Interference Contrast (DIC).Internal signal corresponding to the GPA was not found under standard IFA in the cells either in their undivided (A) or divided state (B) Scale Bar=3µm

C: Immuno-blot analysis of whole cell lysate of erythrocytes, non-infected and infected with *Plasmodium falciparum* and *Babesia divergens*, $4*10^7$ parasite equivalent RBC and iRBC was suitably prepared and the membrane fractions were run on 10% SDS-PAGE gel, followed by semidry transfer and thereafter duly probed with the with anti-Glycophorin A antibody, asterisk sign denotes the bands corresponding to GPA at around 40kDa

However on immuno-gold labelling of high pressure frozen cells infected with *Babesia divergens*, and on IF on Tokuyasu sections of such cells (performed at our collaboration utility at University of Oslo) Glycophorin-A was found internalized and located onto the PVM (Repnik et al., 2015).

A reason behind the failure of this antibody on whole mount labelling but success in labelling of sections could be owing to its size. This commercial antibody being an IgM molecule around

900KDa), it is possible that it failed to enter the cells through the smaller pores generated by our Triton-X-100 mediated permeabilisation and could neither reach the PV crossing the rather thick mesh of fixed cytoplasm nor could enter the lumen of the PV, necessary in order to recognize the epitopes (Krishnamurthy et al., 2007). But while labelling was done on sections, the antibody could have been able to find some of its target epitope, exposed onto the surface and hence was successful in immunolabelling.

3.3.5 Host derived membrane components containing Sialic acid moieties were also found internalized onto the PVM of cells infected by *Babesia divergens*

Erythrocyte invasion by the Apicomplexan parasites *Babesia divergens* and *Plasmodium falciparum* is a receptor mediated interaction those partially depend upon high affinity recognition of sialic acid moieties present on the RBC ligands, such as Glycophorin A and B (Lobo, 2005, Malpede et al., 2013). Proteins or lipids, native to the parasites *Plasmodium falciparum* and *Babesia divergens* are predicted to not to be glycosylated (Macedo et al., 2010). So while a labelling is performed with wheat germ agglutinin (WGA), which specifically binds to Sialic acid (Bhavanandan and Katlic, 1979) moieties and N-acetyl glucosamine (Nagata and Burger, 1974), it stands a good chance to specifically label membrane components those are derived from the host cell.

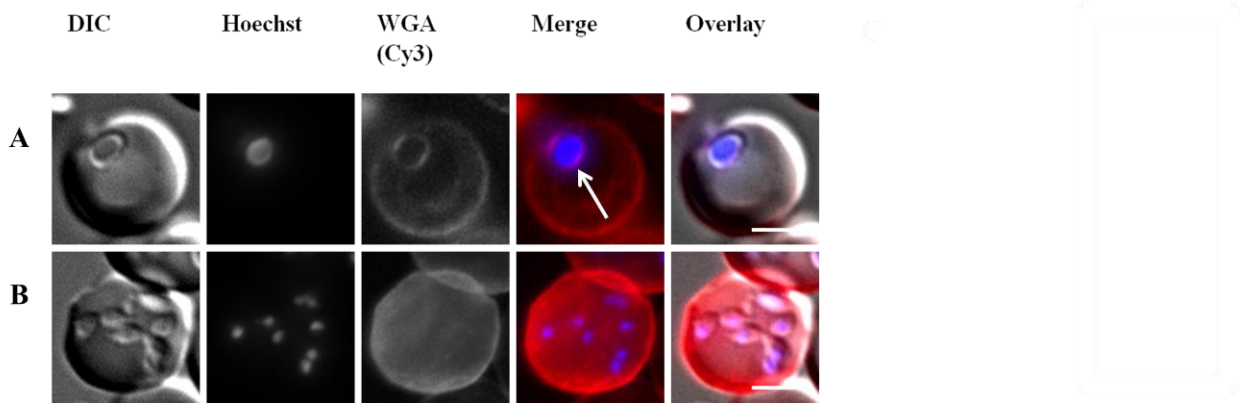


Figure.3.13: Labelling of WGA-binding glycoproteins/glycolipids in erythrocytes infected with *Babesia divergens*

A, B. Whole mount chemical labelling of erythrocytes with WGA followed by *Babesia divergens* invasion and immunodetection. Parasites were identified with Hoechst nuclear staining and with Differential Interference Contrast (DIC) .Internal signal corresponding to the WGA was found in the cells containing undivided parasite (A) but was lost in the ones where the parasite had undergone nuclear division (B) Scale Bar=3µm

After fixing and permeabilisation *Babesia divergens*-iRBC, I performed fluorescence assays (Fig.3.13, A-B) with the WGA on these erythrocytes.

Differentiated merozoite stages of *Babesia divergens* were identified from the undivided Hoechst signals and in a small fraction of such parasites an internal signal of WGA-Rhodamine was found present surrounding (at this resolution) (Fig.3.13A) the Hoechst. Like our previous positive findings, the WGA signal and the corresponding parasite was found closer to the RBCM indicating a recent event of invasion. These finding also confirms the presence of Sialic acid and N-acetyl glucosamine groups in the PVM, and in turn affirms the presence of glycosylated protein components from the RBCM in the PVM. In RBCs, those contained parasites that has already divided and was in their 2 or 4 nuclear stages, no internal signals for WGA-Rhodamine were found irrespective of a stronger surface labelling (Fig.3.13B). The stronger surface signal for WGA in the cells with the later stages of *Babesia* can be a result of a more stabilized membrane organization that is achieved after the initial invasion associated membrane destabilisation had been re-stabilised.

An added advantage of this labelling procedure, owing to the smaller size of WGA when compared to the considerably larger standard antibody molecules IgG (around 150kDa) or IgM (900kDa), is the ease of accessibility of these WGA molecules (38kDa) to the cognate carbohydrate moieties present at the luminal side of the PVM.

3.3.6 GPI anchored and DRM associated proteins of the erythrocyte membrane, Flotillin-1, 2 and CD59 were found on the PMV in the erythrocytes infected with *Babesia divergens*

Recent studies had suggested that GPI anchored and DRM associated proteins CD59, Flotillin-1 and Flotillin-2 are recruited by *Plasmodium falciparum* to different intra-erythrocytic locations and it was inferred that these proteins are associated with the newly formed PVM (Lauer et al., 2000, Murphy et al., 2004). If also found internalized in *Babesia divergens*, these proteins stand a good chance to be used as *bona fide* markers of the PV. For determining the same I carried out immunofluorescence assay on cells infected with *Babesia divergens* and *Plasmodium falciparum* in parallel, using antisera directed against these proteins,.

IFA (Fig.3.14A -L) was carried out using commercial primary antisera against Flotillin-1, (Fig.3.14A -B) Flotillin-2 (Fig.3.14E -F) and CD59 (Fig.3.14I -J) at a dilution of 1:50, 1:50 and

1:100 respectively with a suspension culture of erythrocytes, infected with *Babesia divergens*. The specificity of these antisera were duly evaluated by western blot on lysate of non-infected and infected (*Plasmodium falciparum* and *Babesia divergens*) RBC (Fig. 3.14, C, G, K). It was noticeable that the anti-Flotillin-1 detected more than the expected band corresponding to Flotillin-1 at 48kDa (Fig.3.14C) and the strongest of the bands detected were around 96-98kDa. The commercial antisera used against Flotillin-2 as well detected more than one band at different molecular weights (Fig.3.14G). Apart from a faint band at 42kDa (Fig.3.14G) only distinct in the panels of infected RBC lysate; the antisera detected a more prominent band a higher molecular weight close to 70kDa (Fig.3.14G). These can be a non-specific recognition of the proteins by the commercial antisera but can also be i.e. a diametric form (96-98kDa) of the Flotillin-1 (48kDa protein) (Fig.3.14C) and oligomers of the 42kDa Flotillin-2 protein (Fig.3.14G) detected by the antisera.

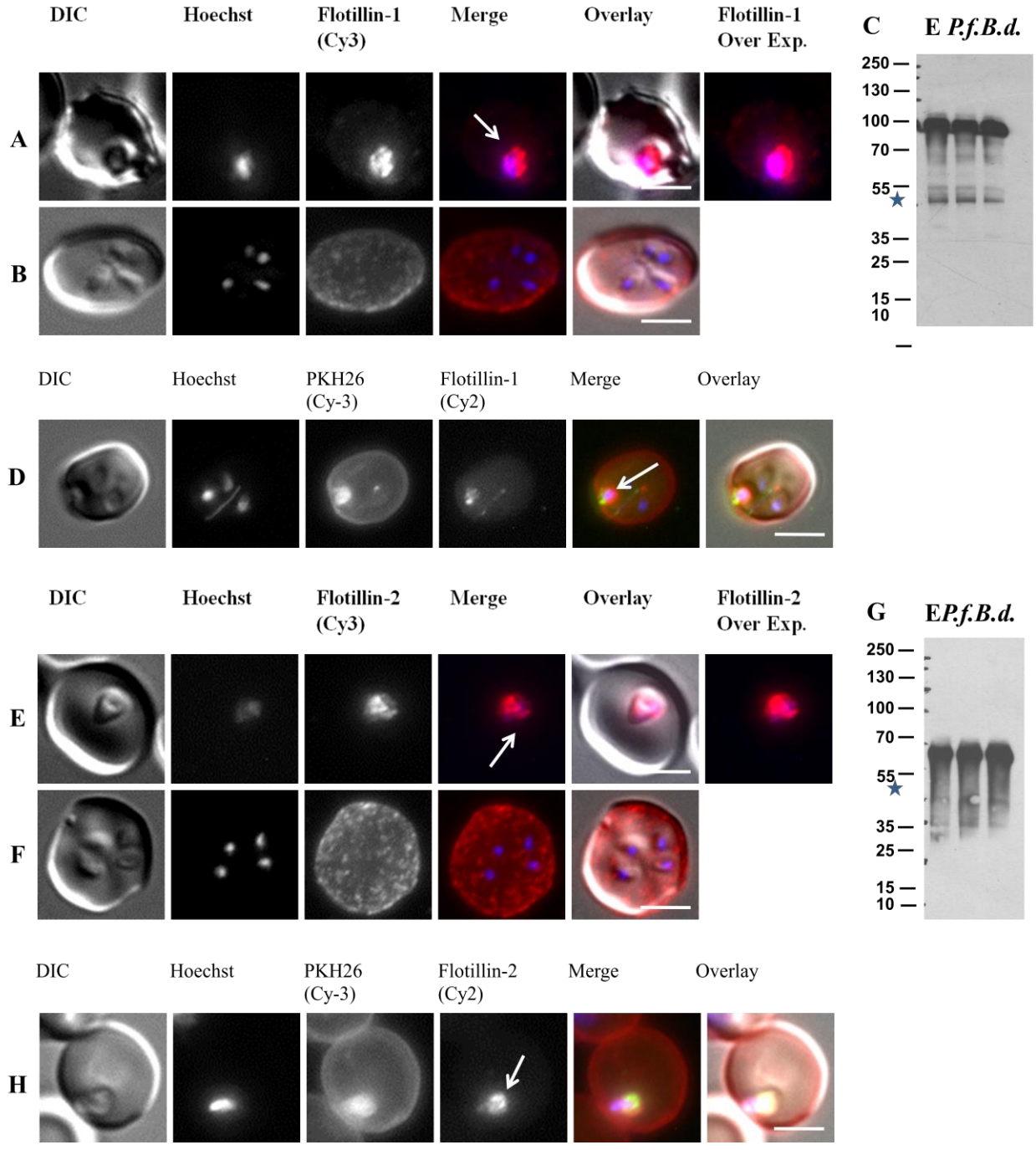
The commercial antibody against CD59 detected a band at around 22kDa corresponding to the CD59 molecule (Fig.3.14K), but it also detected few other larger bands at around 44kDa and 68kDa (Fig.3.124K).The higher bands being approximately in the size of multiples of the CD59 band, it is likely that these commercial antisera is detecting different oligomers of the CD59 protein as well. Unfortunately with the technologies at our disposal oligomeric units of a protein cannot be distinguished differently than the monomer forms of the proteins.

In IFA after incubating the iRBC with the primary antibody and subsequent reaction with a Cy-3 conjugated secondary antibody, these erythrocytes were analyzed under fluorescence microscopy using Hoechst dye for labelling the parasite nuclei.

Under microscope, in cells infected with *Babesia divergens*, the young trophozoites could be seen as a single Hoechst signal (Fig.3.14, A, E, I), representing a single nucleus and the differentiated merozoite stages of the parasite. It was a common observation that such single nucleated cells were found relatively closer to the RBCM, indicating a recent event of invasion. In a fraction of iRBC, with such differentiated merozoites inside, a signal corresponding to Flotillin-1(Fig.3.14A), Flotillin-2 (Fig.3.14E), and CD59 (Fig.3.14I) was found surrounding (at this resolution) the Hoechst signal.

In the matured stages, the parasite appeared divided with multiple individual nuclei arranged opposed to each other or in the typical cross like formation, commonly referred as Maltese cross

(Fig.3.14,B, E, H). No PVM associated signal for Flotillin-1(Fig.3.14B), Flotillin-2 (Fig.3.14F) or CD59 (Fig.3.14J) could be found when the parasite were in such stages. This observation is in line of previous findings indicating disintegration of PVM prior to nuclear division in Babesia spp. (Rudzinska et al., 1976).



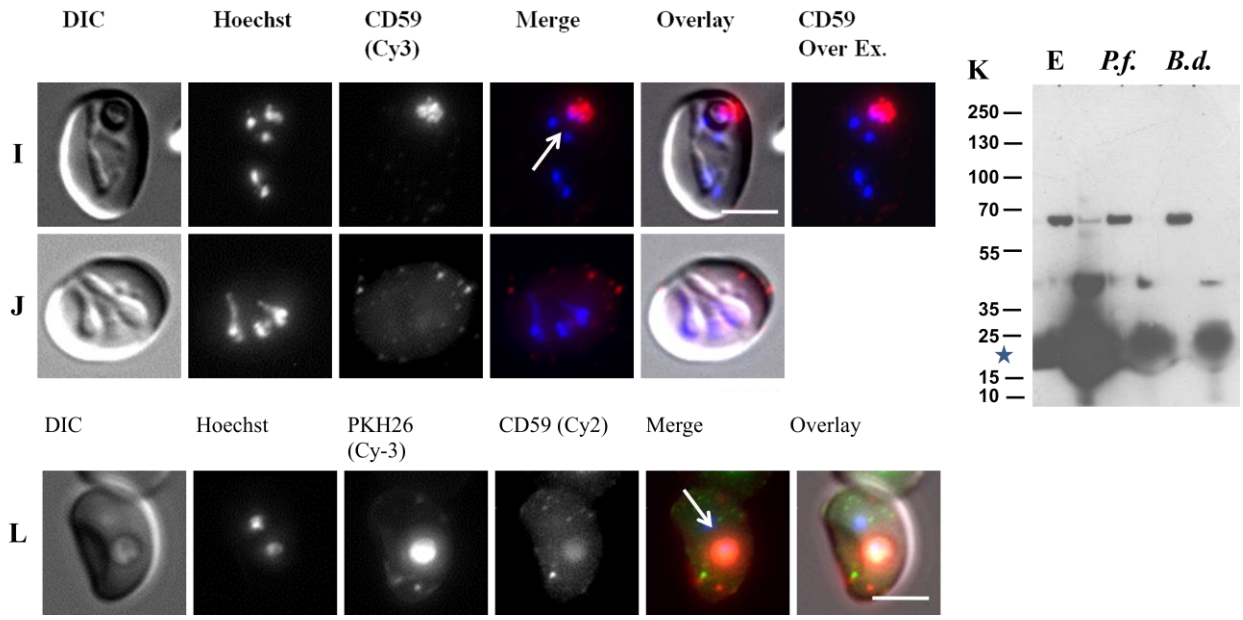


Figure 3.14: Labelling of erythrocytes infected with *Babesia divergens* with Anti Flotillin1, 2 and CD59 antibodies

A, B, E, F, I, J: Whole mount IF labelling of erythrocytes after invasion of *Babesia divergens.* Parasites were identified with Hoechst nuclear staining and with Differential Interference Contrast (DIC).Internal signal corresponding to the Flotillin-1 (A) Flotillin -2 (E) and CD59 (I) was found in the cells where the parasite was in its yet undivided stage whereas in spite of higher surface signal, internal signals were completely lost after the parasites had undergone division (B, F and J). However the signals appeared more global as if staining the whole parasite profile and/or internal structures and were less limited to the periphery. Arrows are representative of the PVM Scale Bar=3µm

C,G,K: Immuno-blot blot analysis of whole cell lysates of erythrocytes, non-infected and infected with *Plasmodium falciparum* and *Babesia divergens,* $4*10^7$ parasite equivalent RBC and iRBC was suitably prepared and the membrane fractions were run on 10% SDS-PAGE gel, followed by semidry transfer and thereafter were probed with anti-flotillin -1(C),Anti-Flotillin 2 (G) and CD59 (K) antibodies .The asterisk signs demark the bands corresponding to Flotillin 1 at 49kDa, Flotillin-2 at 47kDa and CD59 oligomers ranging from 15kDa to 70kDa respectively. For all of the antisera, more than the single band representative of the native protein detected. Noticeably the strongest bands were mostly at higher molecular weight than the desired protein. These bands can be representing oligomeric units of the peptide.

D, H, L: Co localization of internalized PKH-26 (Cy3) with Flotillin-1 (D), Flotillin-2 (H) and CD59 (L).Cy2 denotes the signals corresponding to the erythrocyte membrane proteins and Cy3 stands for the erythrocyte membrane lipids. The Cy2 signal are not limited to the parasite periphery as denoted by the membrane lipids Cy3 indicating the localization of these proteins beyond the PVM. The two signals however are in juxtaposition, to the Hoechst signal representing the single undivided parasite. Scale Bar=3µm

In some instances it could be noticed that some iRBC had been invaded by two or more individual parasites (Fig.3.14I). When invaded at different time points, it was observed that one of those parasites having progressed much through its life cycle had already undergone the cell division and was present as two apposed parasites or as the typical Maltese cross; whereas the

other one was still in its single nucleated stage (Fig.3.14I). In such cases it was noticeable that the signal corresponding to the RBCM proteins was confined only to the single celled stage (Fig.3.14I).

Deliberate over exposure was done to exclude illusion of internal labelling for Flotillin-1 and Flotillin-2 from the cells with multi-nucleated parasites residing inside; hence on such cells, the signal on the RBC membrane appears higher than found in those on the single nucleated stages; which was generally imaged at a lower exposure.

However I could not ignore the fact that the labelling of these parasites with these antibodies was more global than specific (Fig.3.14, A, E and I), thus labelling the whole parasite profile and not only what we know to be the PVM or what we found in case of Band 3 or Spectrin hence I chose to do the immunostaining in presence of the PKH26 stained lipids (Fig.3.14, D, H, L).

For this, the non-infected erythrocytes were labelled with the PKH26 lipid staining kit following the manufacturer's protocol, followed by invasion, immunolabelling and microscopy. The PKH26 being conjugated with Cy3, a Cy2 conjugated secondary antibody was chosen. The labelling confirmed the initial finding and the Cy2 signals corresponding to the Flotillin-1 (Fig.3.14 D)., 2 (Fig.3.14H) and CD59 (Fig.3.14L) antibodies were found, not limited to the PVM denoted by the PKH26-Cy3.They apparently stained more than what the PVM in such infected cells should be like and were staining the whole parasite profile.

The EM analysis using such infected cells confirmed my findings and affirmed the presence of signals corresponding to Flotillin-1, Flotillin-2 and CD59 in the early and uni-nucleated stages of *Babesia divergens* alone and such signal were completely absent in cells, where the parasite had already undergone nuclear division. However like my observations under epifluorescence microscope, under EM as well, the signals for Flotillin-1, Flotillin-2 and to some extent of CD59 appeared more global, staining the parasite cytoplasm and was found less specific to the PVM (Repnik et al., 2015).

Uninfected erythrocytes labelled with the same primary and secondary antibodies showed fluorescence signal associated with and confined to the erythrocyte surface alone. (Fig.3.7). But in parasitized cells, we could observe and rather consistently so, that these commercial antibodies against CD59, Flotillin-1 and 2 reacted strongly to internal structures and not specifically to what we believed to be PVM. This was in-spite of the fact that in western blot

analysis, these antibodies were detecting the corresponding proteins, and possibly their oligomeric forms as well. The reason behind such non-specific staining hence remains enigmatic. This may owe to lack of proper preservation of the disintegrating PVM, or can also be resulting from internalization of the proteins by the parasite, in order to suitably modify them for the PV environment

3.3.7 Multiple-spanning erythrocyte membrane proteins Aquaporin-1 and Aquaporin-3 were also found internalized in erythrocytes infected by *Babesia divergens*

Aquaporin-1 and 3 belong to the group of multi membrane spanning proteins broadly grouped together as Aquaporins; essentially associated with the plasma membrane of eukaryotic cells and involved in the maintenance of cellular homeostasis (Agre, 2006)

Aquaporin-1,which is a rather classical of Aquaporins with major function in and as water selective pores (Agre, 2006), had been shown to be associated with the PVM in erythrocytes infected with *Plasmodium falciparum* in immunofluorescence analysis and had been described to be tentatively associated with DRMs (Murphy et al., 2004).

Aquaporin-3, which is a aquaglyceroporin, with significant permeability for glycerol and moderate permeability for water (Roudier et al., 2002), have reportedly been internalized and located onto the PVM in cells infected with *Plasmodium falciparum* as well but is described to be present in different oligomeric stages (Bietz et al., 2009).

The oligomeric stages shown by Bietz and colleagues are, however different oligomeric stages than those found in their non-infected counterparts (Roudier et al., 2002, Bietz et al., 2009). Acquisition of nutrients, from the iRBC or from the extracellular environment and subsequent release of metabolic waste, is of paramount importance for survival and viability of any parasite. So it will not be surprising if *Babesia divergens* also internalized these proteins and by recruiting those onto their PVM facilitated their nutrient acquisition and export of metabolic wastes. Even during its short existence the PVM should be acting as some barrier between the parasite and the host cell during macromolecular exchange

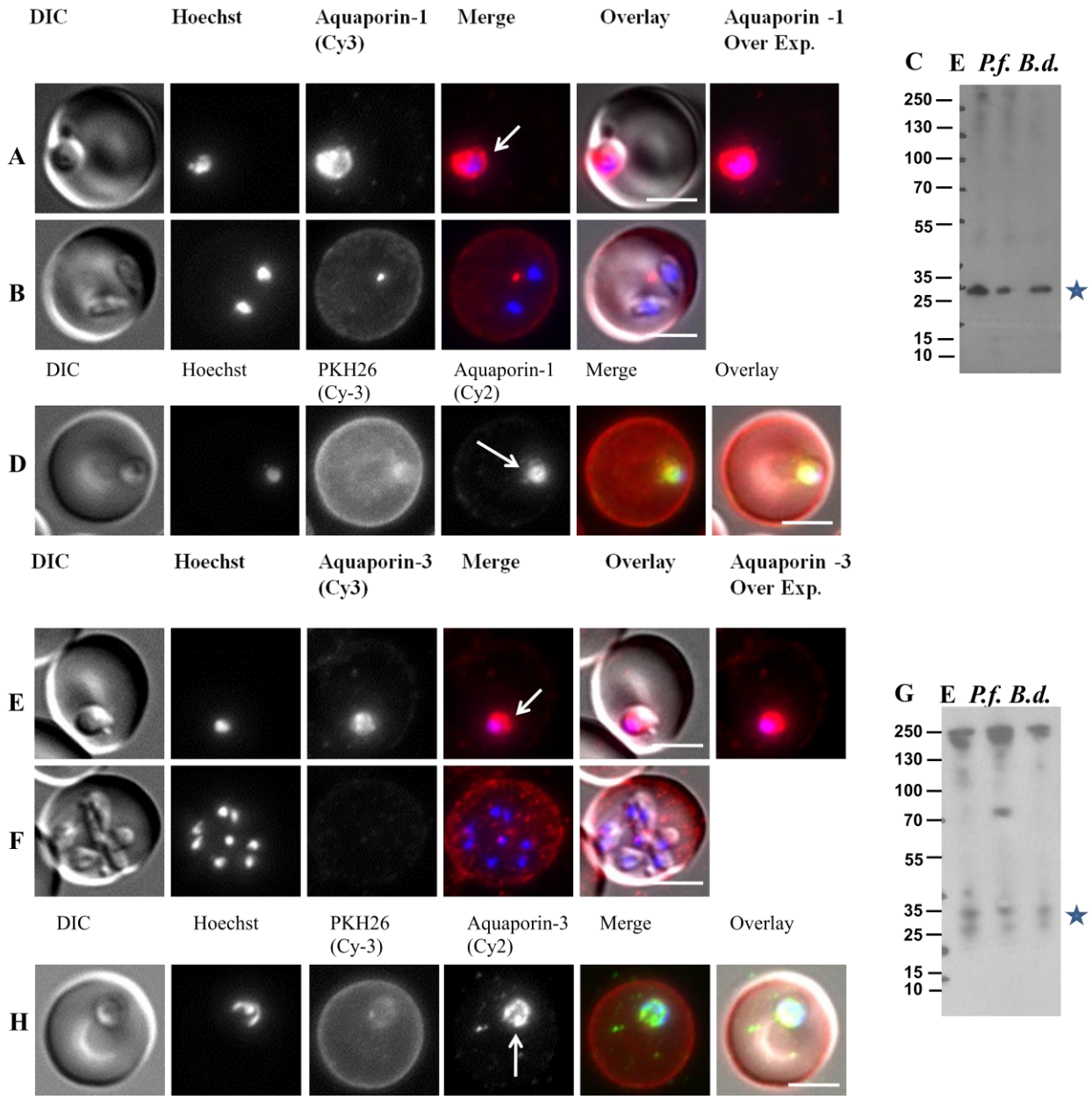


Figure 3.15: Immunolabelling of erythrocytes infected with *Babesia divergens* with Anti-Aquaporin 1 and 3 antibodies.

A, B, E, F: Whole mount IF labelling of erythrocytes invaded by *Babesia divergens.* A parasite were identified by Hoechst nuclear staining and under Differential Interference Contrast (DIC).´Unspecific but internal signal corresponding to the Aquaporin-1 (A) Aquaporin-3 (E) was found in the cells where the parasite was still in its undivided stage, whereas signals were completely lost after the parasites had undergone division (B, F). Scale Bar=3µm

C,G: Immuno-blot analysis of whole cell lysates of erythrocytes, non-infected and infected with *Plasmodium falciparum* and *Babesia divergens* in parallel , $4*10^7$ parasite equivalents RBC and iRBC was suitably prepared. The membrane fractions were run on 10% SDS-PAGE gel, followed by semidry transfer and thereafter were probed with probed with anti-Aquaporin-1(C) and Anti-Aquaporin- 3 (G) antibodies.

The asterisk signs demark the desired bands corresponding to Aquaporin1 at 28kDa and Aquaporin-3 at 32kDa. For both of the antisera, more than the single band representative of the native protein was detected. For Aquaporin-1 only a single larger band was detected © whereas for Aquaporin-3(G) many bands were detected and some bands were specific to parasites. Noticeably the strongest bands were mostly at higher molecular weight than the desired protein. These bands can be representing oligomeric units of the peptide

D, H: Co localization of internalized PKH-26 (Cy3) with Aquaporin-1 (D) and 3 (H) .Cy2 denotes the signals corresponding to the erythrocyte membrane proteins and Cy3 stands for the erythrocyte membrane lipids. The Cy2 signal are not limited to the parasite periphery as denoted by the membrane lipids (Cy3) indicating the localization of these proteins beyond the PVM. The two signals however are in juxtaposition to the Hoechst signal representing the single undivided parasite Scale Bar=3µm.

After immunolabelling with the commercial antisera and secondary antisera-dye conjugate, under microscope, *Babesia divergens*-iRBCs, where the parasite was yet to undergo any division (Fig.3.15, A and E) (denoted by single Hoechst signal corresponding to the nucleus) signals for both Aquaporin-1 (Fig.3.15A) and 3 (Fig.3.15E) could be found, present overlapping (at this resolution) the nuclear signal. Remarkably the signal and corresponding parasites were always found located close to the RBCM. However for both of these antibodies there was neither signal nor significant labelling visible in cells where the nucleus had already undergone division (Fig.3.15, B, and F).

There were many cells with multiple infections (Fig.3.15A). If the infections had happened at different time points, the parasite which had invaded the cell before was found progressed into its 2 or 4 nucleated stages having undergone nuclear division, whereas the parasite that had invaded at a later time point was visible present as a single nucleated entity. In few of such situations, signals corresponding to Aquaporin-1 and 3 were found but these signals were always limited to the parasites in their single nucleated stage. Even in cells where the multiple infections were all at uni-nucleated stages, signals for the Aquaporins was more frequently lost in one but retained in another of such parasites.

After fixing and permeabilising *Babesia divergens* infected erythrocytes, IFA were carried out (Fig.3.15, A, B, E, F) using commercial antibodies directed against Aquaporin-1 (Fig.3.15A- B) and Aquaporin-3 (Fig.3.15E-F) Prior to the IFA analysis the specificity of these commercial antisera were determined by western blot analysis (Fig.3.15, C and G). The commercial Anti Aquaporin-1 antibody was found to be specific and detecting a single band at around 28kDa corresponding to the Aquaporin-1 peptide (Fig.3.15C). The commercial Anti Aquaporin-3 antibody however detected several bands at 30-32kDa (Fig.3.15G) (non- infected and infected RBC lysate), 35kDa (lysate of non infected and infected RBC), 70kDa (lysate of *Plasmodium*

falciparum infected RBC), 100kDa (non-infected RBC), 220kDa (non infected and infected RBC lysate) and 250kDa (non infected and infected RBC lysate).

Aquaporin-3 exists in oligomeric forms in the non-infected erythrocyte and another set of oligomeric forms of Aquaporin-3 are also reported in erythrocytes infected with *Plasmodium falciparum*. The different bands in my western blot analysis here might correspond to these oligomeric states of the Aquaporin-3 peptide detected by the anti-Aquaporin-3 antibody in lysate of infected and non-infected erythrocytes.

However for these proteins as well, it was noticeable that the immuno-staining pattern was less restricted to be like a borderline, what that could be denoting the PVM and was more overall the parasite (Fig.3.15, A, E) as if reacting with the parasite cytoplasm. This observation was further strengthened while IF was carried out on thin sections, where the whole profile of the parasite was lit and not the typical pouch like staining of PVM could be found as expected (performed at Oslo).

To address this discrepancy, I performed immunolabelling with the Anti Aquaporin antibodies on PKH26 labelled-*Babesia* infected erythrocytes. Non-infected erythrocytes were labelled with the PKH26 lipid staining kit following the manufacturer's protocol, followed by invasion, immunolabelling and microscopy. The PKH26 being conjugated with Cy3, a Cy2 conjugated secondary antibody was chosen for the immunostaining.

Immunolabelling of these PKH26 labelled- *Babesia divergens* infected erythrocytes confirmed the initial observations and Cy2 signals corresponding to the Aquaporin-1(Fig.3.15D) and 3 (Fig.3.15H) antibodies were found not limited to the PVM denoted by the PKH26-Cy3.The Aquaporin antibodies apparently stained more than what the PVM in such infected cells should be like. This again can be a non-specific reaction of the antibody, but it seems more plausible that the Aquaporins are being internalized by the parasite therefore when the antibodies are detecting these internalized Aquaporin, in their different oligomeric forms.

Under EM, my observations about the incorporation of Aquaporin 1 and 3 onto the PVM in erythrocytes infected with *Babesia divergens* were further confirmed. But under EM as well the anti Aquaporin 1 and 3 antibodies were found to be labelling the whole parasite profile and structures inside the parasite, instead of a pouch like staining characteristic to the PVM (Repnik et al., 2015) like was observed from immunostaining with (Band 3 and spectrin) marker or in

lipid staining. Thus it seems that these antisera, used for the labelling were not specific for what is believed to be the PVM and instead were labelling the whole parasite.

3.3.8 Unlike in *Babesia divergens* infected erythrocytes, in *Plasmodium falciparum* infected erythrocytes, Band 3, Spectrin, Glycophorin and/or WGA were not found to internalized and/or recruited onto the PVM

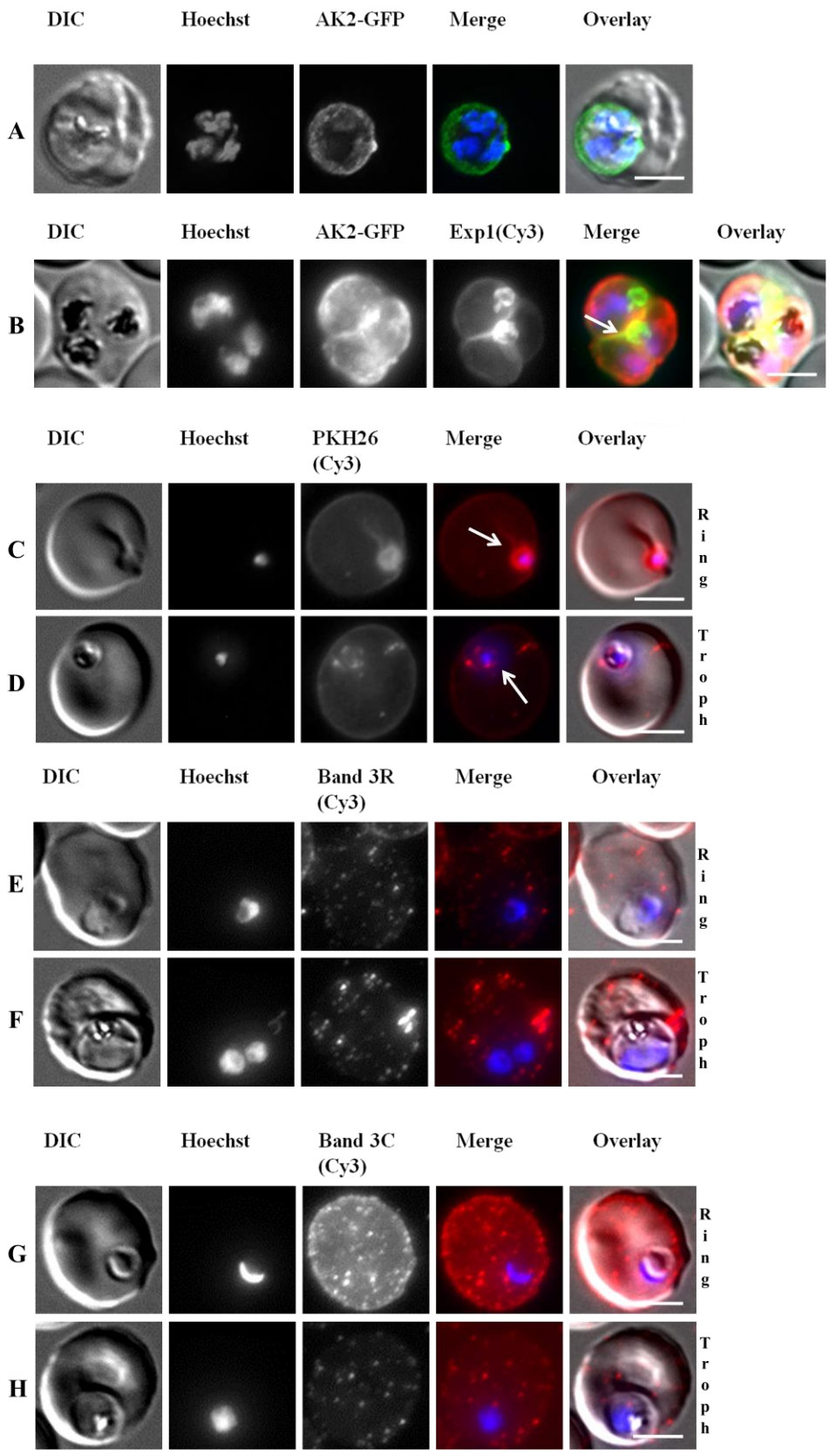
The existing concept about compositions of the newly formed PVM, advocates about a selection amongst the erythrocyte membrane proteins during their recruitment onto the newly forming PVM and is based on data, majorly obtained from studying *Plasmodium falciparum* infected erythrocytes.

According to the working hypothesis the high copy number proteins and transmembrane proteins of erythrocyte are not recruited to the PVM because of their cytoskeleton association whereas a set of erythrocyte membrane proteins containing GPI anchors and/or associated with the membrane raft domains are taken up in what seems to be a selective process.

But my recent findings in *Babesia divergens* infected erythrocyte shows association with cytoskeleton did not hamper recruitment of a high copy number trans-membrane proteins onto the PVM in *Babesia* spp. and moreover cytoskeletal protein itself was found onto the PVM of *Babesia divergens* against the cytoskeleton association to be any . My results with wheat germ agglutinin further suggest an incorporation of host cell glycoprotein and glycolipids during the formation of the PVM in Babesia divergens infected erythrocytes.

Hence I decided to reinvestigate the compositions of the PVM in erythrocytes infected with *Plasmodium falciparum* to address the disparity (Fig.3.16A-N).

To distinguish what the PVM specific immuno-staining should be , I used an already established transfectant parasite line of AK2-GFP that expresses a PVM localized GFP chimera (Fig.3.16A) (Ma *et al* 2012) and used an antibody against PfEXP-1 (*Plasmodium falciparum* exported protein-1) (1:100) as a control for the PVM exported proteins itself (Fig.3.16B). As a control for the internalization of lipids in the PVM of *Plasmodium falciparum*, I used the PKH26 membrane lipid staining kit and protocol (Fig.3.16C-D) used successfully in my experiments with *Babesia divergens.*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Hoechst
AK2-GFP
Merge
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A
DIC
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AK2-GFP
Exp1(Cy3)
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DIC
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PKH26 (Cy3)
Merge
Overlay
C
Ring
D
Troph
DIC
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Band 3R (Cy3)
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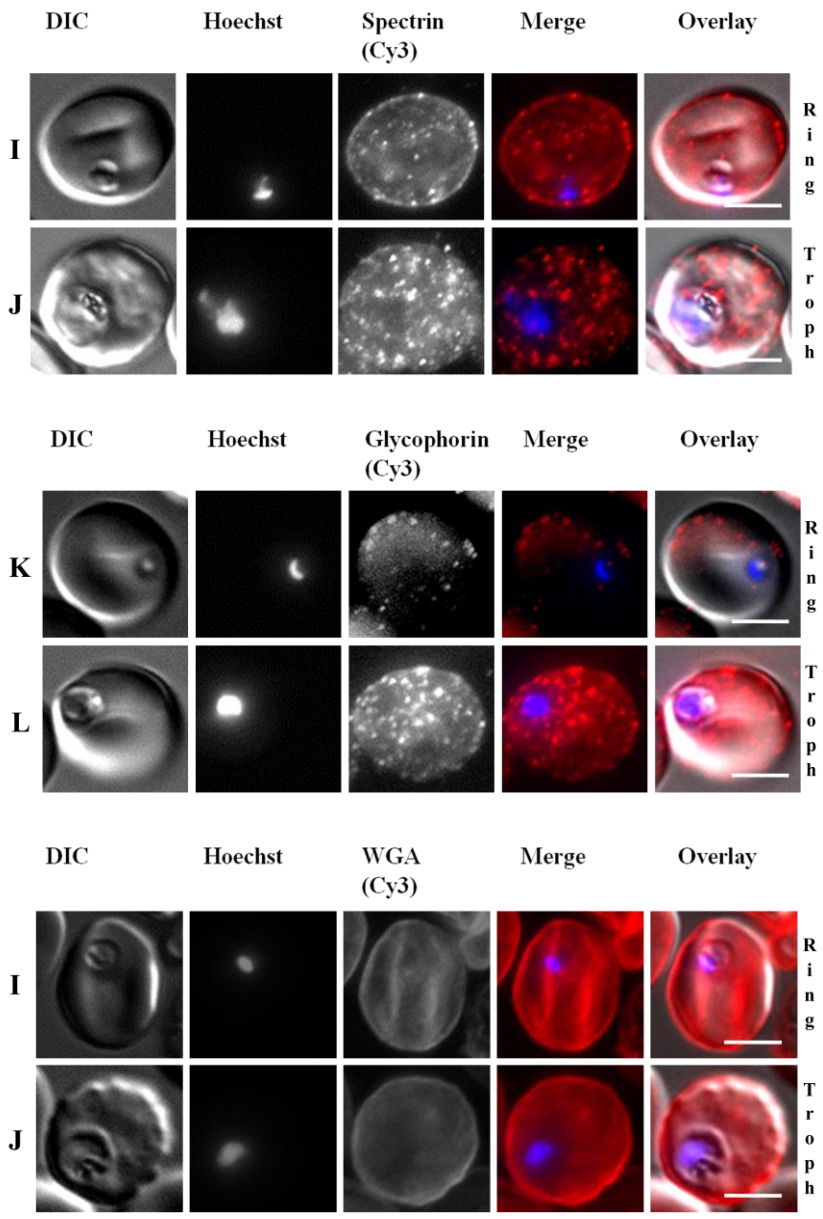


Figure 3.16: Whole mount fluorescence labelling of RBC infected with *Plasmodium falciparum* with the parasite in its different stages of development (Rings, trophozoites and Schizonts) as indicated in the legends

A-B: Images of cells infected with AK2-GFP transfectant line of *Plasmodium falciparum* Scale Bar=3µm

B: Co-localization of GFP chimera of AK2-GFP and PfEXP1 on the PVM of *Plasmodium falciparum*-iRBC

C-N: Images of erythrocyte infected with wild type parasites on which IF were performed. Scale Bar=3µm

Independently both the AK2-GFP transfectant (Fig.3.16A-B) and PKH26 (Fig.3.16C-D) labelling indicated a ring like staining pattern representing the PVM surrounding the Hoechst signal representing the parasite, in erythrocytes infected with *Plasmodium falciparum* AK2-GFP

(Fig.3.16A-B) and 3D7 cell lines respectively (Fig.3.16C-D). The PVM localization of the AK2-GFP chimera itself was further confirmed by IFA on duly fixed permeabilized *Plasmodium falciparum* AK2-GFP infected erythrocytes with the anti *Pf*EXP-1antibody (Fig.3.16B). Such PVM staining however was completely absent in the cells infected with *Plasmodium falciparum* with the parasite in either the rings and trophozoites stages, when immunofluorescence assays were performed with antibodies against Band 3 *i.e.* Band 3R (Fig.3.16E-F) and Band 3C (Fig.3.16G-H), Spectrin (Fig.3.16I-J), Glycophorin (Fig.3.16K-L) and WGA (Fig.3.16M-N). On repeated experiments and control experiments these observations being constant, I did not attempt to co-localize these antibodies with the AK2-GFP transfectant lines. But it was notable that a much stronger labelling of erythrocyte surface was observed in cells infected with *Plasmodium falciparum* than was observed in cells infected with *Babesia divergens* with these antibodies in general. Under EM as well no specific signals for Band 3 (Band 3R and Band 3C), Spectrin, Glycophorin and/or WGA were found, corresponding to the PVM in cells infected with *Plasmodium falciparum* in either of their ring or trophozoite stages (Repnik et al., 2015).

3.3.9 GPI anchored, DRM associated proteins Flotillin-1, 2 and CD59 could be seen onto the PVM of *Plasmodium falciparum* infected erythrocytes

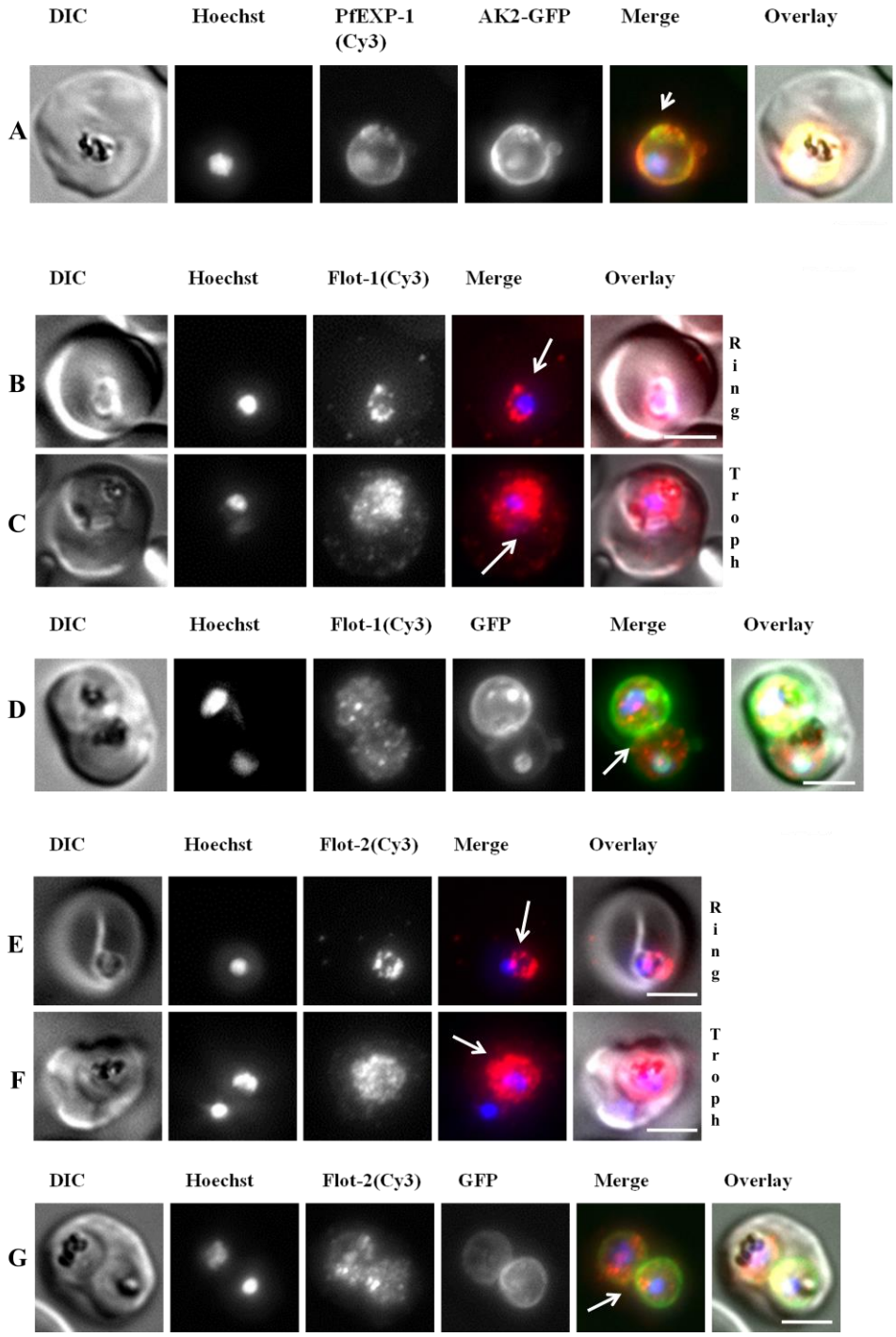
Several of the erythrocyte membrane detergent resistant micro domain associated proteins had reportedly been form on the PVM of *Plasmodium falciaprum*. My immunofluorescence analysis with *Plasmodium falciparum* infected erythrocytes showed findings similar to the earlier reports suggesting the presence of Flotillin-1, Flotillin-2 and CD59 on the PVM in both ring and trophozoite stages of the parasites (Fig.3.17,B-C, E-F, H-I).

However I could not ignore the fact that the labelling of parasites especially in their trophozoite stages was more global than specific and there was labelling of the whole parasite profile and not only of the PVM should be.

To illustrate the difference between the typical PVM staining to those found with these antibodies, AK2-GFP transfectant line was used as a control. IFA was performed on these chimerical cell line (Fig.3.17, D, G and J) in parallel to *Plasmodium falciparum* 3D7 strain infected erythrocytes (Fig.3.17, B-C, E-F, H-I).

It could be clearly seen that the immunostaining pattern with the commercial antisera against the proteins like Flotillin1, 2 and CD59 exceeded the rim like GFP staining pattern of AK2-GFP

(Fig.3.17, D, G and J) and stained profiles of the cell that seemed to be parasite cytoplasm. The *Pf*Exp-1 was used furthermore as a control of the PVM localization of the AK2-GFP chimera itself (Fig.3.17A)



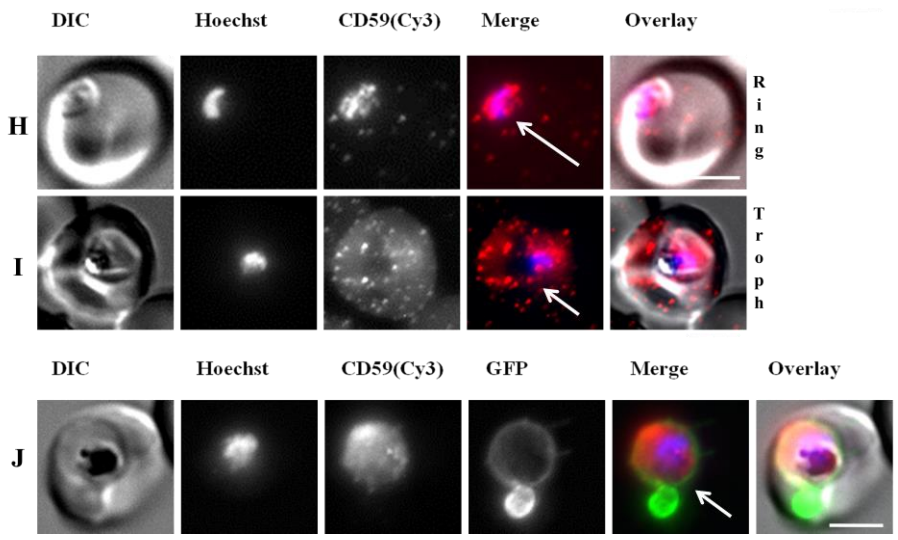


Figure 3.17: Whole mount fluorescence labelling of RBC infected with various stages of *Plasmodium falciparum* (Rings and trophozoites) as indicated in the legends and control of PVM staining with *Pf*EXP-1 antibody on AK2-GFP transfectant line of parasites

A Co-localization of the PVM targeted GFP chimera of AK2-GFP and *Pf*EXP-1 onto the PVM of the cells infected with *Plasmodium falciparum,* as control of PVM associated staining. Images of cells infected with AK2-GFP transfectant line. B-P images of erythrocyte infected with wild type *Plasmodium falciparum* 3D7 (B, C, E, F, H, I, K, L, N, O) and AK2-GHP chimera (D, G, J, M, P) those were further immunolabelled with specific antibodies as mentioned in the legends Scale Bar=3µm

EM analysis with erythrocytes infected with *Plasmodium falciparum* with the parasites in both of their ring and trophozoite stages confirmed our findings about the presence of signals corresponding to Flotillin-1, Flotillin-2, and CD59 but could not be conclusive about the signals corresponding to the PVM. Similar to our observations under epifluorescence microscope, under EM as well, the signals for Flotillin-1, Flotillin-2 and to some extent of CD59 appeared more global, staining the whole parasite cytoplasm and less specific towards the PVM itself (Repnik 2015). These antibodies seemingly were reacting to some internal structures in the parasitized cells and we could observe strong signals around the whole parasite in both of the ring and trophozoite stages. This is of the fact that the antibodies against Flotillin-1, 2 and CD59 were detecting their corresponding peptides their different oligomers in western blot analysis (Fig.3.14 C, G and K)

3.3.10 Multiple membrane spanning protein of erythrocyte membrane, Aquaporin 1 and were found onto the PVM of *Plasmodium falciparum*.

Several reports in had shown an incorporation of the protein from the Aquaporin family on the PVM *Plasmodium falciparum* of infected erythrocytes. I tested the results for two of the Aquaporins namely Aquaporin 1 and 3 on erythrocytes infected with *Plasmodium falciparum*.

In IFA (Fig.3.18A-F) on cells infected with *Plasmodium falciparum*, signals corresponding to Aquaporin-1 (Fig.3.18A-C) and aquaporin-3 (Fig.3.18D-F) were found overlapping (at this resolution) the corresponding Hoechst signal in both the ring or trophozoite stages of the parasite. However noticeable was the fact that the staining for both Aquaporin 1 and 3 especially in the later stages of the parasites, was more overall as if it was reacting with the parasite cytoplasm and much less specific towards the PVM (Fig.3.18B, E)

This observation was further strengthened while IF was carried out on AK2-GFP transfectant lines and the Aquaporin antibodies were used label these GFP-chimera expressing cell. For both the antibodies the staining was beyond the delineation of PVM staining as represented by GFP. The complete profiles of the parasite was seemingly immuno labelled (Fig.3.18C-D) and not the typical pouch like staining could be found as was seen for only AK2-GFP (Fig.3.12A) and/or for Exp-1(Fig.3.17A) co-labelling with AK2-GFP (Fig.3.17B)

EM analysis with *Plasmodium falciparum* infected erythrocytes containing the parasites in both of their ring and trophozoite stages confirmed our findings about the ‘presence’ of signals corresponding to Aquaporin 1 and Aquaporin 3 but EM too could not give any conclusive results about the localization Aquaporin 1 or 3. However like our observations under epifluorescence microscope, under EM as well, the signals for these antibodies were seemingly global and appeared to be the whole parasite cytoplasm and less specific towards the PVM itself.

In the parasitized cells, we could observe strong interaction of these antibodies to the internal structures of the parasite and not specific interaction to what we believed to be PVM. This is in-spite of the fact that the antibodies were found to be detecting the proteins and their different oligomers in western blot analysis.

Moreover my observations from the co-localization experiments using AK2-GFP transfectant line and these commercial antisera, poses another question about the authenticity of the accepted results about the internalization and recruitment of these proteins onto the newly formed PVM

in *Plasmodium falciparum* infected erythrocytes. Most of the previous researches had used these commercial antisera and have reported such proteins to be internalized and recruited onto the newly formed PVM of *Plasmodium falciparum*. My findings show a possibility of internalization but questions about the specific recruitment as advocated by the contemporary researches so far. This question hence remains open and requires for further research.

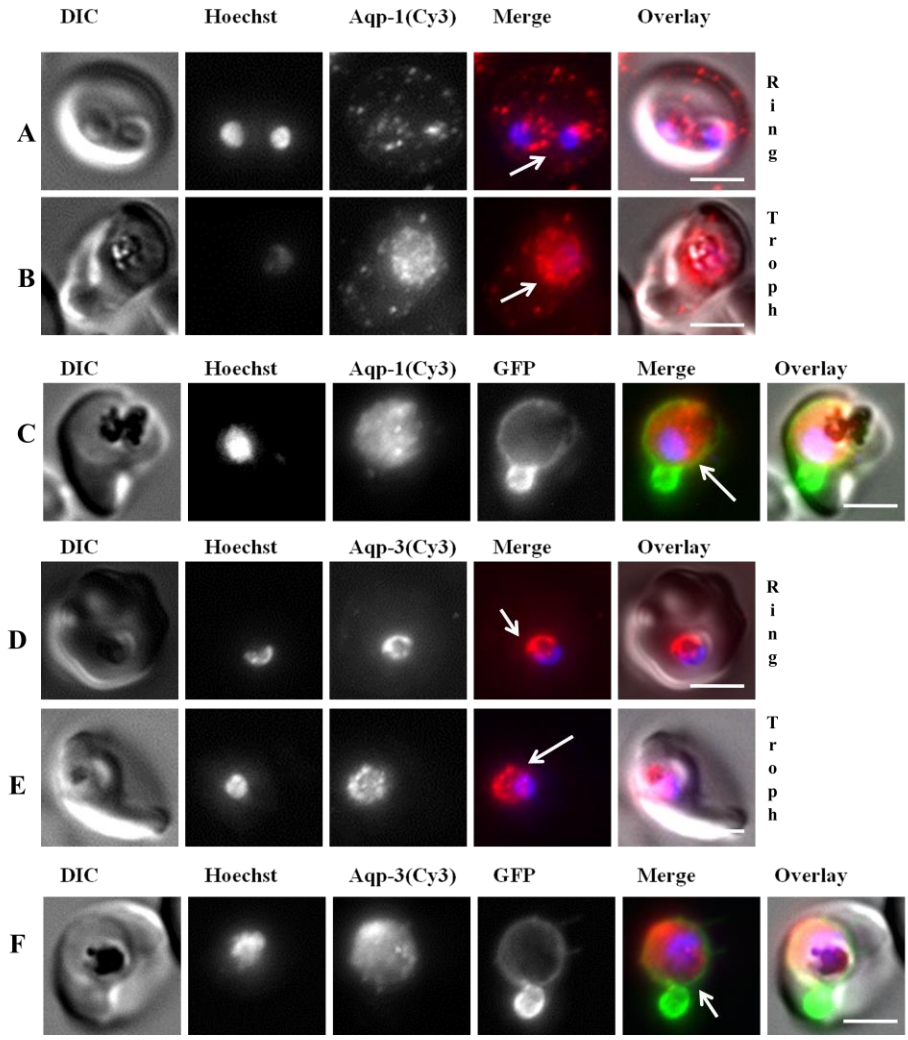


Figure 3.18: Whole mount fluorescence labelling of RBC infected with various stages of *Plasmodium falciparum* (Rings and trophozoites) as indicated in the legends and control of PVM staining with PfEXP-1 on AK2-GFP transfectant line of parasites

A Co-localization of the PVM targeted GFP chimera of AK2-GFP and PfEXP-1 onto the PVM of the cells infected with *Plasmodium falciparum*, as control of PVM associated staining. Images of cells infected with AK2-GFP transfectant line. B-P images of erythrocyte infected with wild type *Plasmodium falciparum* 3D7 (B, C, E, F, H, I, K, L, N, O) and AK2-GHP chimera (D, G, J, M, P) those were further immunolabelled with specific antibodies as mentioned in the legends Scale Bar=3µm

3.4 Confirmation of the accuracy of the immunodetection process

The secondary antibodies I used were either Cy2 or Cy3 conjugates and to test their inertness on labelling erythrocyte membranes or parasite components by themselves, I checked their specificity by IFA on the iRBC and non-infected RBC. The secondary antibodies were found not to be reacting alone to any of the membrane proteins (Figure 3.19A-D)

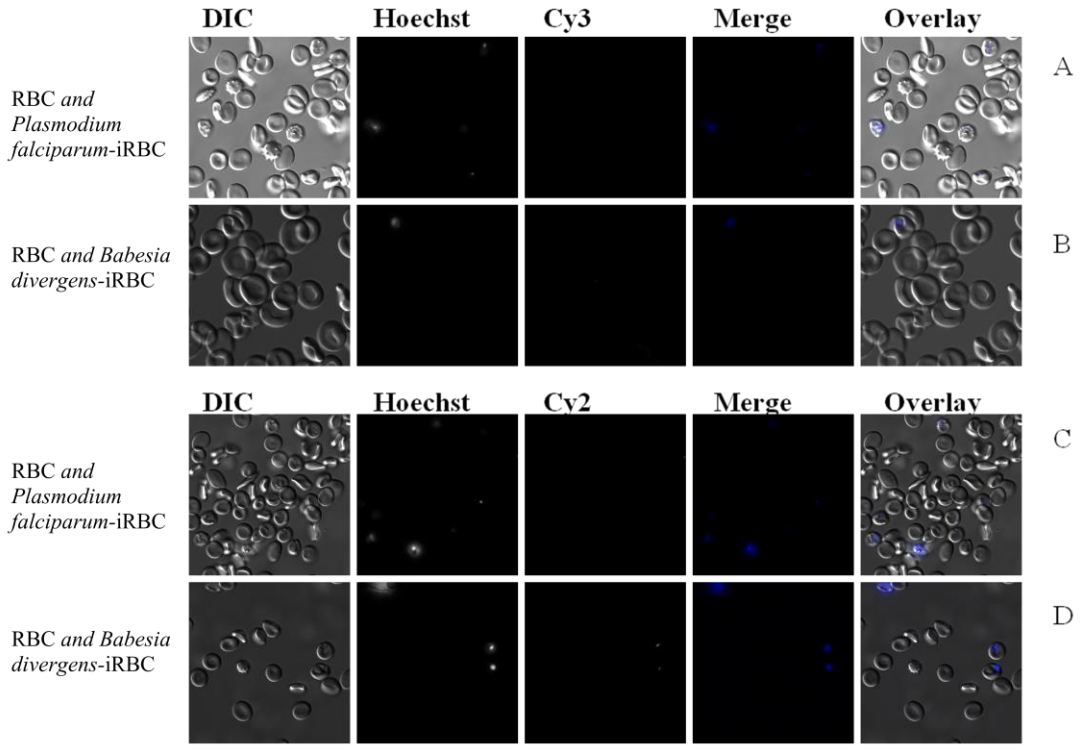


Figure 3.19: Whole mount immunofluorescent labelling of *Plasmodium falciparum* infected and *Babesia divergens* infected RBC with secondary antibodies

RBC and iRBC were fixed, permeabilized, blocked against non specific interaction and were incubated overnight with 3% BSA dissolved in PBS (pH7.4) at 4^{o}C. On the following day the cells were washed and were incubated with Cy2/Cy-3-conjugated secondary antibodies and were incubated for 2 hours at room temperature. Parasites were identified with DIC and Hoechst nuclear staining under microscope. Neither the Cy-3(A, B) conjugated secondary antibody, nor the Cy2(C, D) conjugated secondary detected any membrane structures in absence of suitable primary antibody, in non-infected and *Plasmodium falciparum* and/or *Babesia divergens* infected erythrocytes.

I have further controlled the immunodetection and to eliminate the chances of any technical artefacts related to non-specific labelling. I immunolabelled non-infected or infected erythrocytes with a protein known to be generally absent in erythrocyte named Ty-Tag (Fig.3.20A). Ty-Tag is a 10-amino-acid sequence from the immunologically well-characterised major structural protein of the Ty1 virus-like particle of *Saccharomyces cerevisiae* and is thus not expressed in RBC. On

immunolabelling the non infected and infected erythrocytes I could show Ty-Tag does not label RBC (Fig.3.20A).

Thereafter I co-immunolabelled the *Babesia divergens* infected erythrocytes for Band 3 and Spectrin, in presence of the non-specific Ty-Tag. This experiment showed that anti Ty-Tag antibody did not co-localize with internal Band 3 (Fig.3.20B) or Spectrin signal (Fig.3.20B), representing the PVM. And in turn it strengthens the accuracy of the experimental results.

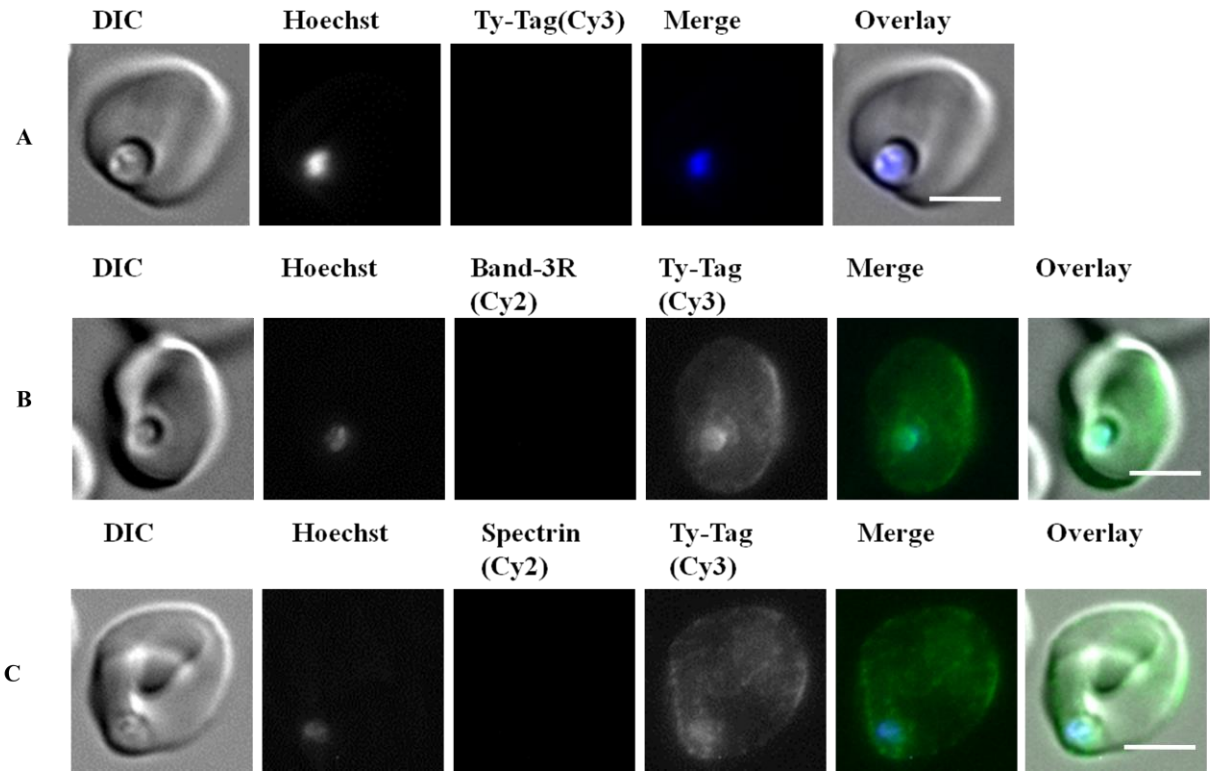


Figure 3.20: Whole mount immunofluorescent labelling of *Babesia divergens* infected RBC with anti-Ty-Tag alone or together with anti-band 3 or anti-spectrin.

Rabbit monoclonal Band 3 and rabbit polyclonal Spectrin antibodies were used at 1:100, followed by Cy2-conjugated goat anti-rabbit secondary antibody. Mouse monoclonal anti-Ty-Tag antibody was used at 1:50, followed by Cy3-conjugated goat anti-mouse secondary antibody. Parasites were identified with DIC and Hoechst nuclear staining. Scale Bar=3µm

4 Discussion

During invasion, many of the intracellular pathogens (bacterial and eukaryotic alike) utilize the phagocytic and endocytic properties of their respective host cells and reside secluded, inside a phago-lysosomal vacuolar compartment (Haas, 2009). However, the Apicomplexan protists have evolved a set of complex machinery that enables them to enter their respective host cells by an active process (Sibley, 2004, Soldati-Favre, 2008). Conceivably, such an active form of invasion broadens the range of hosts that could be exploited. Majority of the apicomplexan are obligate intracellular parasites and many can invade nucleated cells, whereas the enucleated human erythrocytes are host to comparatively smaller number of apicomplexan, including *Plasmodium* spp. and *Babesia* spp. A few other apicomplexan parasites (e.g. *Cryptosporidium* spp., *Theileria* spp.) occasionally invade the erythrocytes [reviewed in (Sibley, 2011, Plattner and Soldati-Favre, 2008)]. However, the erythrocytes do not seem to play any essential or obligatory role in the course of these infections, as they do for malaria (plasmodial infection) and babesiosis (babesial infection).

From the perspective of cell biology, the active invasion of mammalian erythrocytes, by these obligate intracellular parasites, is of significant interest as well. Apart from being terminally differentiated, erythrocytes are incapable of endocytosis and/or phagocytosis and thus are incapable of taking up any parasite on its own (Burns and Pollack, 1988). The highly specialized mesh-like cytoskeleton underlying the erythrocyte membrane and anchored to it via various proteins (Smith, 1987) make the active invasion for these parasites even more complex. These facts, put together with the limited nutritional and metabolic resources available within the erythrocyte, presumably are the apparent reasons behind such a narrow spectrum of parasites the mammalian RBCs can serve host for. Therefore this selection of the mammalian erythrocyte as an obligatory host implies a higher level of adaptation to this unusual environment, on part of these parasites (*Plasmodium spp.* and *Babesia spp*).

The process of active invasion is quintessentially a receptor-ligand mediated interaction (Soldati-Favre, 2008) and the receptors present on the red blood cell surface with their respective ligands on the parasites have been studied in details; but cells infected with of *Plasmodium falciparum* had been mostly under focus (Cowman and Crabb, 2006). But the molecular mechanisms underlying structural alterations of RBCM during parasite invasion and/or the contributions of

the host cell membrane components towards these alteration processes and in turn towards the formation of PVM remain unclear.

Once inside the erythrocyte and thereafter all along the development until egress, *P. falciparum* remains within its PV delineated by the PVM secluding it from the erythrocyte cytosol, Several hours after invasion, appearance of extensions had been reported in the cytosol of such infected erythrocytes (Bannister and Mitchell, 2003). Eventually formation of an extensive membranous network of Maurer's clefts and tubulovesicular membrane had been found in these infected erythrocytes (Wickert and Krohne, 2007, Lauer et al., 1997). But whether these networks are all interconnected and/or are connected to the PVM is a matter of debate in itself (Wickham et al., 2001, Hanssen et al., 2008, Wickert and Krohne, 2007).

The PVM is a large membrane and is estimated to be around 30-30μm^2 ,yet is formed *de novo* in 10-20seconds (Suss-Toby et al., 1996). In erythrocytes infected with *P. falciparum*, the lipids both from the erythrocyte membrane and from the parasite apical organelle are known to contribute towards the PVM formation (Aikawa et al., 1978, Aikawa et al., 1981, Ward et al., 1993, Pouvelle et al., 1994). As the malarial infection progresses, *P. falciparum* synthesizes an array of integral membrane proteins and lipids and exports beyond the parasite periphery; some of which are retained within the PV (Nyalwidhe and Lingelbach, 2006) and/or are inserted into the PVM (Gunther et al., 1991, Spielmann et al., 2012) whereas others traverse the PVM *en route* to their final destinations (Ansorge et al., 1996). Synthesis and export of protein and lipid components by the parasite is known cause major alterations in the PV environment altogether (Maier et al., 2009).

Over the last decade many individual researches had shown recruitment of erythrocyte membrane lipids (Ward et al., 1993, Dluzewski et al., 1992) and proteins onto the PVM of *P.falciparum.*(Lauer et al., 2000, Murphy et al., 2004, Bietz et al., 2009). It is therefore plausible that the lipids and proteins from the erythrocyte membrane are also involved in the formation and subsequent extensions of the PVM and for other membranous networks, found inside the infected erythrocytes; the latter being a process supposedly driven by the metabolic activities of the parasite (Elmendorf and Haldar, 1994).

To understand the contributions on part of the parasite and the host cell during the formation and in extension of the unique intra-cellular structure of PVM, I did a comparative analysis of the PVM formed during the invasion of two related apicomplexan parasites.

I chose to study the recruitment of erythrocyte membrane proteins and lipids onto the newly formed PVM in cells infected with *Plasmodium falciparum* and *Babesia divergens.* By comparing the recruitment of membrane components by these two parasites to their respective PVMs, I aimed to achieve a better understanding of what that is common in such invasions, thus can be accredited to the host cell and what is unique and therefore should be accreditated towards the parasite.

4.1 Elucidating the ultrastructure of *Babesia divergens* infected erythrocyte

Much work had been carried out to elucidate the ultrastructure of PV and the components PVM in erythrocytes infected with *P.falciparum*. Majority of the information available about such compartmentation in *Babesia* spp. is from the study of Rudzinska and colleagues on *Babesia microti* infected murine/non primate erythrocytes (Rudzinska, 1976).Neither much is known about the gross ultrastructure of the *Babesia divergens* infected erythrocytes nor about the ultrastructure of the vacuolar compartments like PV and/or the delineating PVM in such erythrocytes.

Should the host cell be contributing towards vacuolar compartmentation like PV and affecting the inclusion and/or exclusion of specific components (lipids, proteins) onto the PVM, similar host cell components should be found recruited or discounted from the PVM of different parasites that infect the same host cell. With this analogy, I designed and executed subsequent experiments and EM analysis with *Babesia divergens*, adapted to human erythrocytes. I used *Babesia divergens* isolate from human patient (Gorenflot et al., 1998); as apart from helping us to understand the gross ultrastructure of the vacuolar compartments in *Babesia* divergens-iRBC, it allowed me compare the cell biology of *Babesia* divergens-iRBC to those of *Plasmodium falciparum*-iRBC in subsequent experiments. As both of the parasites (*Plasmodium falciparum* and *Babesia divergens*) were maintained and propagated in human erythrocytes obtained from identical donors, I could do a direct comparison between any invasion-induced alterations.

Our observations with *B. divergens* were in similar line to what was reported in earlier studies by Rudzinska et al, (1976). In erythrocytes infected with *B. divergens* PVM was found only in a

very small fraction of cells, where the *B. divergens* was found as a single entity and had not had undergone any nuclear division. In some of such erythrocytes, vesicular structures were observed near the PVM, indicating a probable membrane blebbing resulting from the membrane disintegration event (Repnik et al., 2015).

The erythrocytes with trophozoite and/or differentiated merozoite stages of *B.* divergens, no vacuolar compartmentation was found. In these stages, the parasite appeared surrounded by a single membrane (parasite plasma membrane) and was in direct contact with the host cell cytosol. The later intra-erythrocytic stages of the *B. divergens*, where the parasites had already undergone cell division, these were found present as 2-4 individual nuclei, arranged in distinct patterns (Repnik et al., 2015) like reported from the observations from Rudzinska et al (1976).

A reason why the vacuolated stages of the *Babesia divergens* were rare to find in the electron microscopic sections, may owe to the fact that the disintegration of the PVM was rather difficult to preserve and the disintegration process itself is fast (Asada et al., 2012). In contrary under IFA it was easier to locate and distinguish such single nucleated stages of *B. divergens* as the incidence of associated antibody labelling was easier to notice and thereafter to characterize. Apparently the remains of the PVM of these transient stages were better revealed by antibody labelling both under epifluorescence microscopy and EM (immuno-gold labelling) than by just ultrastructure analysis. The quick disintegration of the PVM and lack of proper preservation of the disintegrating PVM may be one of the reasons why the fluorescence staining in my later experiments often appeared clustered along the parasite rather than a rim-like staining delineating the vacuolar membrane.

The erythrocyte invasion by apicomplexan parasites is completed in different steps (Sibley, 2004, Soldati-Favre, 2008). Fundamentally, after the initial attachment with target cells, the parasite re-orients itself bringing its apical end into juxtaposition with the host cell plasma membrane. It is followed by discharge of the apical organelles creating an indentation on the membrane and the rapid invagination of the plasma membrane concomitant to the formation of the PV. Several individual studies had demonstrated that, at the site of contact of the *P. falciparum* and the erythrocyte, underneath the erythrocyte membrane electron dense structure termed as ‘tight junction’ is formed; a formation reportedly mediated by ligand-receptor

interaction bringing the invading parasite plasma membrane and the erythrocyte membrane together (Soldati-Favre, 2008, Sibley, 2004).

However in our EM analysis on *B. divergens*-iRBC, in the invading stages of the parasites no such tight junction could be found. This is in spite of the fact that *Babesia divergens* codes for a protein, homologous to the *P.falciparum* Apical Membrane Antigen-1; (Montero et al., 2009, Tonkin et al., 2013, Jackson et al., 2014).- a protein known to be essentially involved in the tight junction formation during invasion of *Plasmodium* spp.(Sharma and Chitnis, 2013, Tyler et al., 2011)

In *Theileria parva* another Apicomplexa, a unique form of invasion is found, where after initial contact of merozoite with the host lymphocyte a zippering mechanism seals the incoming parasite with the host cell enclosing it in a vacuolar compartment without calling for any tight or moving junction (Shaw, 2003, Webster et al., 1985).In another Apicomplexa Cryptosporidium spp. somewhat different cell invasion mechanism can be found where the host cell microtubule associated proteins rearrange and cause microvillus like extensions of the host enterocyte membrane which gradually surrounds the parasite and fuses along its borders to form a non-fusogenic vacuole delineated by a PVM(Wetzel et al., 2005, Plattner and Soldati-Favre, 2008). However an electron dense ring beneath the surface of the parasite, at the site of contact is reported and presumably beneath this the host actin are depolymerised by parasite kinases (Chen et al., 2004).Parasite kinase mediated actin de-polymerization and host cell microtubule rearrangement is key in such invasion(Forney et al., 1999). But in absence of tubulin in erythrocyte cytoskeleton such a mechanism seems unlikely mediating the invasion of *Babesia divergens*.

Therefore it calls for a thorough analysis to understand and confirm if the apparent absence of the tight junction, governing the invasion of *Babesia divergens* is a fundamental difference of an essential structure in Apicomplexans (Besteiro et al., 2011) or should this be attributed to the very small number of invading *Babesia* that we could observe, therefore we could not find any.

Unlike *P. Falciparum*, no morphological criterion (like change in parasite size, appearance of food vacuole) distinguishes between the young and progressively older single cell stages of *Babesia divergens.* However the later stages of *Babesia* spp. duly termed as differentiated

trophozoites and piriform (pear shaped) merozoites are distinguishable due to their shapes and paired or Maltese cross like organization (Rudzinska et al., 1976, Lobo et al., 2012).

It is also possible that some of the invading *Babesia* do not form a PVM at all and thus lack any internalized proteins. Nevertheless, host cell invasion is one of the fundamental cell biological processes and not having a PVM formation during invasion is seemingly unlikely. Instead, it is possible that the single nucleated parasites, which lack in PVM, are the more developmentally progressed ones. From these observations it could be certainly said that in contrast to the *Plasmodium falciparum*, which retains the PVM all along the intra-erythrocytic development, in *Babesia divergens* infected erythrocytes; disintegration of PVM precedes any nuclear and/or cellular division. Thus, the loss of PVM can be a useful criterion in itself to distinguish younger single cell stages of the parasite from the progressively older ones

These observations put together is also provide conclusive evidence about the fact that the compartmenting membrane seen under EM are actually representative of the newly formed PVM in cells infected with *Babesia divergens* and it secludes at least the young stage parasites from the host cell cytosol (Repnik et al., 2015). However, at the same time, this leaves us with an open question about when after invasion does the PVM actually disintegrate.

4.2 Synchronisation of erythrocytes infected with *Babesia divergens:* identification of the time of PVM disintegration

In *B. divergens*-iRBC the nuclear division giving rise to the later stages of trophozoites and piriform merozoites, succeeds disintegration of the PVM. These stages are distinguishable under light microscopy due to their unique shapes and typical arrangement in patterns inside the erythrocytes, termed as Maltese cross (Lobo et al., 2012, Rudzinska et al., 1976). However under simple light microscopy, no apparent morphological difference was detectable between the early and progressively older single nucleated stages of *Babesia divergens*.

Under EM, we found a fraction of these single nucleated stages, surrounded by a vacuolar membrane. Therefore, we reasoned these cells to be the earlier of the single nucleated stages of *Babesia divergens* whereas the fraction of *B. divergens* found surrounded by the parasite plasma membrane alone, lying in direct contact with the erythrocyte cytosol, were reasoned to be the relatively later of the single nucleated stages.

To understand the formation, components and disintegration of the PVM in *B. divergens* we needed more of these early single celled stages of the parasite. However, the lack of morphological demarcation made getting such stages more complex.

Apart from clear morphological differences between the early (ring) and later (trophozoite) stages of *Plasmodium falciparum*, the later stages of the parasite are also associated with different morphological and cell biological alterations of the infected erythrocytes. By exploiting these altered biophysical and biochemical properties of the *P. falciparum*-iRBC, different synchronisation techniques been developed to preferentially enrich the desired stages of the parasites in culture, like Gelafundin floatation (Pasvol et al., 1978), Sorbitol treatment (Lambros and Vanderberg, 1979) and by applying high intensity magnetic field (Paul et al., 1981).

Similar morphological and biochemical alterations had been reported in erythrocytes infected with *Babesia divergens* (Alkhalil et al., 2007, Aikawa et al., 1992, O'Connor and Allred, 2000). I used the chemical and physical methods already well established in synchronizing *Plasmodium falciparum*-iRBC and made several attempts to synchronise the development and preferentially enrich the desired stages of *Babesia divergens*. My assumption was that in absence of morphological differences amongst the early stages of the parasite, these altered biophysical and biochemical properties the erythrocyte itself could be helpful in isolating the preferred stages. However, these experiments did not yield any positive result in *B. divergens*-iRBC (Table 3.1,3.2 and 3.3; Page No.70-71).

One possible explanation for this can be, a possible difference between mechanisms governing the morphological and cell biological alterations in *Plasmodium falciparum*-iRBC to those governing in *Babesia divergens*-iRBC.

In later stages of the erythrocytes infected with *Plasmodium falciparum*, knob like protrusions develop beneath the erythrocyte membrane (Luse and Miller, 1971). These ‘knobs’ are involved in altering the cytoadherence properties of such infected cells and in turn are involved in mediating the cell sequestering (Leech et al., 1984). Formation of these knobs however also alters the buoyancy of these erythrocytes and when treated with Gelafundin, the altered buoyancy helps in isolation of the older and more buoyant Trophozoite stages from the younger ones (Pasvol et al., 1978).

Ridge-like membrane protrusions akin to the plasmodial knobs are reported in .cells infected with *Babesia spp.* (Parrodi et al., 1989, Hutchings et al., 2007). *Plasmodium falciparum*-iRBC, show an increased cytoadherence possibly mediated by the increased affinity of the 'knobs' towards the cell surface ligand CD36 (Cooke and Coppel, 1995). However in a key experiment Hutchings and colleagues in 2007 while describing the molecular details underlying the formation of ridges *Babesia spp.*-iRBC demonstrated that unlike found in the *Plasmodium*, the *Babesia*-iRBC had no such increased affinity towards CD36 (Hutchings et al., 2007).

Self-aggregation of KAHRP, beneath the surface of the erythrocytes infected with *P. falciparum* is known to be a major modulator in the formation of knobs on such cells (Chishti et al., 1992, Aikawa et al., 1986, Kilejian, 1979); however, no homolog of *Pf* KAHRP was found in the *Babesia* spp. (Hutchings et al., 2007). The ridges of *Babesia* spp. infected erythrocytes were found to be sensitive towards trypsin (Hutchings et al., 2007) much unlike the trypsin tolerance of the 'knobs' of *P. falciparum* infected cells (Luse and Miller, 1971).

These put together most certainly indicate towards a different mechanism regulating the membrane dynamics in *Babesia divergens* infected erythrocytes and is a likely reason behind the failure of Gelafundin mediated isolation of iRBC containing older stages of the parasites.

Nutrient supply, both in terms of variety and quantity, is limited within the confines of the erythrocytes. The invading parasite therefore principally depends on the erythrocyte cytosol and the haemoglobin and sometimes on the surrounding media for acquisition of its essential nutrients. *Plasmodium falciparum* engulfs the haemoglobin through its cytostome, in a phagocytosis like process (Krugliak et al., 2002, Lew et al., 2003, Barry, 1982). As the parasite remains surrounded by the PVM, the haemoglobin is engulfed together with some erythrocyte cytosol, in form of packets, covered by two membranes with the inner membrane representing the PVM (Elliott et al., 2008). During the intra-erythrocytic development phases, *Plasmodium falciparum* digests approximately 75% of the total haemoglobin (Lew et al., 2003, Loria et al., 1999).Thereafter it is degraded by the parasite proteases, in a lysosome like compartment duly termed food vacuole (Ragheb et al., 2011). The peptic degradation products of haemoglobin are used up by the parasite whereas the indigestible 'Haem' fraction polymerizes and accumulates as Haemozoin. In the later developmental stages (early to late trophozoite, early schizonts) of *P. falciparum* the haemozoin accumulate is deposited inside the food vacuole. This accumulation of

ferromagnetic Haem complex is exploited to isolate the later stages of the iRBC, as in the presence of high intensity magnetic field, these Haemozoin containing stages are temporarily arrested whereas the younger stages flow through (Paul et al., 1981). Haemoglobin serves as the major nutrient supply to the *Babesia divergens* as well; however, the details of mechanisms behind the uptake and the degradation of haemoglobin in *Babesia* are not well-understood (Rudzinska, 1976, Langreth, 1976). I tried to synchronise *B. divergens* by applying high intensity magnetic field like done in *P. falciparum* spp but my attempts of isolating later stages of *Babesia divergens*-iRBC were ineffective.

A possible reason behind this can be the lack of Haemozoin or Haemozoin like complex formation and/or subsequent accumulation in *Babesia divergens*. *Babesia* do not have a typical cytostome and it is known that in *Babesia* the haemoglobin is not taken up by any phagocytosis like process either (Barry, 1982, Langreth, 1976). Over the years, extensive research with different species of *Babesia* has established involvement of different sets of proteolytic enzymes in the degradation of haemoglobin (Barry, 1982, Wright and Goodger, 1973, Mesplet et al., 2010, Shenai et al., 2000). It was also demonstrated that in comparison to *Plasmodium berghei* the haemoglobin degradation in *Babesia rodhaini* is a much slower process (Barry, 1982) , suggesting a thorough removal of degradation products. Another hypothesis however favours the presence of a fundamentally different haemoglobin degradation pathway in *Babesia* in comparison to the *Plasmodium* (Rudzinska, 1976, Simpson et al., 1967).

In either context the haemoglobin degradation in *Babesia divergens*-iRBC is a seemingly a more thorough process and is devoid of any food vacuole formation or formation of any residual haemozoin pigment (Langreth, 1976, Rudzinska, 1976, Kawai et al., 1999, Richier et al., 2006) and in absence of any/substantial accumulation of any ferromagnetic or diamagnetic compound inside the infected cells, the magnetic field was ineffective in isolating any specific stage of the parasite.

Erythrocytes infected by *Plasmodium falciparum* and *Babesia divergens;* both are known to show altered permeability to various solutes during advanced stages of the infection (Ginsburg et al., 1983, Kirk et al., 1994, Alkhalil et al., 2007, Desai and Rosenberg, 1997). Sorbitol a sugar-alcohol, exploits the altered permeability to penetrate cells and cause osmotic imbalance leading to the lysis of such cells (Lambros and Vanderberg, 1979). Erythrocytes containing the younger

stages of the parasites, being less permeable to Sorbitol undergo no osmotic imbalance and hence remain intact. Enrichment of the ring stages of *Plasmodium falciparum* is achieved by treating them with 5% Sorbitol solution and the results are highly reproducible.

However, my several attempts to synchronise *Babesia divergens* infected erythrocytes using 5% Sorbitol solution did not succeed. Treatment with Sorbitol failed, negligible lysis of *Babesia divergens*-iRBC whatsoever and even when a decreasing (5%-1%) or increasing (5%-10%) concentration of Sorbitol was used or other experimental conditions (Standard 10 minutes at 37^0 Celsius) like incubation time (1min-10minutes), incubation temperature (4^o Celsius), and/or centrifugation speed were altered the cells were intact. The reason behind this is beyond my understanding.

A major aim behind synchronizing the *Babesia divergens*-iRBC was to identify and closely monitor the formation and the point of degradation of the PVM, and this I could achieve by (at least partially) doing a time course analysis on live PKH26 labelled iRBC under microscope. On following the fate of single *Babesia divergens* after invasion of the PKH26 lapelled erythrocytes, I could confirm that the PVM originated from the erythrocyte membrane and detached from the membrane surface within 5-8 seconds after invasion. It was not possible to understand more intricate details of PVM formation under epifluorescence microscopy hence if there was any involvement of the *Babesia* spp. rhoptries or was there any formation of a tight junction remained unclear.

I optimized the experimental procedures to gain access to more of these newly infected erythrocytes, where the PVM would still be intact. I allowed a controlled contact between non-infected erythrocytes and highly parasitized (80%-90%) *Babesia divergens* infected erythrocytes and this enabled some lateral infection. The infection rate was low but was moderately synchronous and the synchrony was retained during the initial 2-3 cycles akin to a transient enrichment; allowing me access to -more of the single nucleated stages of *Babesia divergens*-iRBC.

However, it needs more work to ascribe a time point to the disintegration of PVM specifically and for overall synchronisation of erythrocytes infected with *Babesia divergens*, to understand any stage specific internalization of erythrocyte membrane proteins or expression of parasite proteins.

4.3 Understanding the contribution of erythrocyte membrane proteins towards the formation of PVM

In distinction to any phago-lysosomal compartments, the invading merozoites of *Plasmodium falciparum* (like other Apicomplexans) reside in the intracellular compartment of parasitophorous vacuole (PV) being surrounded by a single unit membrane termed as parasitophorous vacuole membrane (PVM). During ultrastructure analysis with *Babesia divergens*, under EM we could see that it also forms a PV during invasion and remains at least temporarily arrested in it before the PVM disintegrates, leaving it in direct contact with erythrocyte cytosol.(Repnik et al., 2015).

We could also see vesicular structures in the vicinity of the PVM in *B. divergens* ,indicating a possible membrane blebbing supporting membrane disintegration.(Repnik et al., 2015); similar structures were also reported in studies by Rudzinska and colleagues (1976). But no membrane bound structural extensions in the cytosol of the erythrocytes could be found suggesting of the presence of any TVN like compartmentations. Reportedly the mechanisms and molecules used by the invading apicomplexan parasites are conserved, (Maier et al., 2009), but whereas the *Plasmodium falciparum* remains secluded in the PV all during its erythrocytic phases in *Babesia divergens* the compartment was found to disintegrate. Many proteins transported onto the surface of *Babesia* infected erythrocytes seemingly are not involve in pathways like what is found in *P.falciparum* infected cells (Allred et al., 2000, Jackson et al., 2014). These observations indicate towards the fact that the maintenance of PV per se as a protective environment or as a sorting compartment is not essential at least in the *B. divergens.*

The erythrocyte being incapable of any receptor mediated endocytosis (Haberman et al., 1967, Singer and Morrison, 1976) ; formation of the membrane bound vacuolar compartment like PV is a cell biological enigma in itself and not much is clearly understood about its maintenance and/or subsequent disintegration as well. It is known that *P.falciparum* exports an array of proteins to modulate the PV environment (Maier et al., 2009) but the contributions on part of the erythrocyte in general are still unclear.

It has been implied that the association with erythrocyte membrane cytoskeleton is prevents the incorporation of some of the erythrocyte membrane proteins in the PVM of *Plasmodium*

falciparum whereas some proteins are recruited onto the PVM based to their association with these microdomains (Ward et al., 1993, Lauer et al., 2000, Murphy et al., 2007)

In my IFA experiments with *Babesia divergens*-iRBC (Fig.3.14, Fig. 3.15), and also with *Plasmodium falciparum*-iRBC (Fig.3.17,Fig.3.18), I could see signals corresponding to GPI anchored and DRM associated erythrocyte membrane proteins like Flotillin-1, Flotillin-2, CD59 and Aquaporin-1 and also for membrane spanning protein like Aquaporin-3.

In both of the ring and trophozoite stages in *Plasmodium falciparum*-iRBC the signals corresponding Flotillin-1, 2 and CD59 (Fig.3.17,B-C,E-F,H-I) and for Aquaporin-1 and 3 (Fig.3.18,A-B,D-E) were found associated with the parasite; which is how it should be as the PVM is retained in *P.falciparum* during all of the intra erythrocytic phases.

In *Babesia divergens*-iRBC however the signals corresponding to Flotillin-1 (Fig.3.14A), 2 (Fig.3.14E) and CD59 (Fig.3.14I) and for Aquaporin-1 (Fig.3.15A) and 3 (Fig.3.15E) were found in only a fraction of the single nucleated differentiated merozoite stages of the parasite [(Fig.3.14, A, E, I; Fig.3.15, A, D)]. The signal was completely lost from the stages where the parasite had undergone division (Fig.3.14 B, E, H; Fig.3.15,B, E).This observation further substantiates the idea that in *Babesia* divergens the PVM is lost soon after invasion and the disintegration precedes the nuclear or cellular division of the parasite.

However, the labelling for these marker proteins both in *Plasmodium falciparum* and in *Babesia divergens* infected erythrocytes appeared different from our understanding of a PVM localized expression. The immunolabelling for these markers in both of the parasites appeared more global staining the whole parasite and was not limited to the periphery of the parasite; typical of a PVM specific expression. The discrepancy in PVM staining of *Plasmodium falciparum* was further confirmed when I compared the immunolabelling for these markers in the backdrop of a known PV resident-GFP fused protein expression (Fig.3.17, D, G, J and Fig.3.18, C and F).

I used a well-established transfectant line of AK2-GFP (Ma et al., 2012) that expresses a PV targeted protein-GFP chimera and performed the IFA for the erythrocyte membrane proteins with the backdrop of this chimerical GFP expression. The GFP signal appeared rim like surrounding the parasite profile (Fig.3.16A) and was found delineated to the parasite periphery, whereas the immunostaining for the markers appeared not limited only to the parasite periphery (Fig.3.17, D, G, J,) and (Fig.3.18, C and F,).

To authenticate the expression of this PV resident-GFP fusion marker itself I compared the GFP expression with the immunolabelling for another well established PV targeted protein PfEXP-1 control (Fig.3.17A). The GFP signal and immunolabelling for PfEXP-1 both appeared rim like delineating the parasite periphery akin to the PVM (Fig.3.17A).

For *B. divergens*, I compared the immunostaining for these proteins (Flotillin-1, Flotillin-2, CD59, Aquaporin-1 and Aquaporin-3) (Fig. 3.14, D, H, L, Fig. 3.15, D and H respectively) in the background of internalized PKH26-Cy3 labelled erythrocyte membrane lipids (representing the PVM). The internalized PKH26 appeared rim like, surrounding the parasite profile representing the PVM; and not staining the whole parasite, whereas the markers appeared not limited only to the parasite periphery (Fig.3.14, D, H, L, Fig.3.15, D and H respectively). The secondary antibodies alone did not react to the parasites (Fig.3.19). There were no non-specific recruitment of proteins onto PVM and I confirmed this by immunoassaying for a random protein Ty-Tag known to be absent in erythrocyte. (Fig.3.20)

In the ultra-structure analysis under EM, we noticed that the disintegration of PVM in *B. divergens* was fast and the PVM was difficult to preserve. Such a poorly preserved disintegrating PVM could be a reason behind the overall staining in *B. divergens*. However, so cannot be the reason in *Plasmodium* as it retains the PVM all along its intra erythrocytic phases. Moreover, the lack of residual membrane fragments or associated staining in the later stages of *Babesia divergens* infected erythrocytes needed explanation as well.

On the basis of my IFA analysis it was obvious that a substantial amount of the marker proteins were getting accumulated in or around the *Plasmodium falciparum* and *Babesia divergens*. Immunoblotting analysis with the lysate of these infected erythrocytes further confirmed the presence of these proteins in the membrane fraction. However during the immunoblotting all of these commercial antisera, detected more bands at different molecular weights than the one at the specific molecular weight.

An easy but possible explanation for this can be non-specificity of the commercial antisera used for the immunodetection. However, the basis of such immunodetection is recognition of specific epitopes by the commercial antibodies, only this specificity is commercially tested, and stringently quality controlled. In the proteolytic environment of the parasite interior, if the erythrocyte membranes proteins are internalized and thereafter processed, the occurrence of

oligomers of the native protein and/or protein degradation products containing the same epitopes highly increase. With the methodologies at our disposal, such products will not be distinguishable from the native protein. Thus, the possibility that the antibodies are detecting different oligomers of the same protein or are detecting some proteolytic products of the proteins cannot be completely overruled either.

In erythrocyte infected with *Plasmodium falciparum,* Aquaporin-3 is known to be internalized immediately during or after the invasion and is not transported in retrograde to the PVM (Bietz et al., 2009). It in the lysate of such infected erythrocytes the protein was also detected in different oligomeric stages than were reported to be found in the non-infected cells by Roudier and colleagues (Roudier et al., 2002, Bietz et al., 2009). The association of Flotillin-1, Flotillin-2, and CD59 and of Aquaporin-1 to the membrane microdomains, along with their incorporation to the PVM (probably during invasion) had been much discussed (Lauer et al., 2000, Salzer and Prohaska, 2001, Murphy et al., 2004). But their specific role in nutrient transport and in turn in the development of the parasite inside erythrocyte remains much unclear. It is possible that like Aquaporin-3, these proteins as well are not retrogradely recruited from the erythrocyte membrane onto the PVM like was hypothesized by Lauer and colleagues (2000) and Murphy and colleagues in (2004) but instead are internalized by the invading parasites *Plasmodium falciparum* and *Babesia divergens* (Lauer et al., 2000, Murphy et al., 2004).

Many of the proteins secreted by the *Plasmodium falciparum* and found present in the PV space are known to be proteases (Nyalwidhe and Lingelbach, 2006, Dowse et al., 2008).Therefore it is a possibility that following the internalization of the erythrocyte membrane associated proteins , these parasite proteases alter the oligomerization of these proteins, modifying them suitably for the PV environment prior to recruiting them onto the PVM.

It would be beyond the topic of my thesis and a topic of independent research in itself to study the importance of these proteins in the infected erythrocytes, and thereafter to identify if there indeed is an internalization induced change in oligomerization of these proteins to customise them for the PV environment.

While I performed IFA with antisera for major RBCM proteins like Band 3, Glycophorin and erythrocyte cytoskeletal protien Spectrin, the observations in *Plasmodium falciparum*-iRBC was exclusion of these major erythrocyte membrane proteins from the PVM (Fig.3.16E-N),

seemingly confirming hypothesis in practice suggesting exclusion of such proteins owing to their biophysical properties and association with erythrocyte cytoskeleton (Ward et al., 1993, Dluzewski et al., 1992, Murphy et al., 2004, Bietz et al., 2009).

When IFA was performed on *Babesia divergens*-iRBC with the same antisera, the results were different. In a small subset of erythrocytes where the invading merozoite had undergone differentiation but no nuclear division was evident , signals corresponding to the anti Band 3 antibodies i.e. Band 3R (Fig.3.8, A, C) and Band 3C (Fig.3.8, A, C) ,and also for anti-Spectrin (Fig.3.11A) were found to be associated with the parasite periphery. Such signal was completely missing from the cells where the parasite had undergone division and was present as 2-4 individual entities (Fig.3.8, B and D), (Fig.3.9, B and D) and (Fig.3.11B).With two different antibodies, directed against the N and C-terminal of the Band 3 peptide respectively, the observations could be reproduced and confirmed.

In a different experiment, (Fig. 3.10), when the same antibodies were suitably incubated with a synthetic (commercial) peptide designed to block the recognition (of N-terminal region of Band 3R), the anti-Band 3R antibody failed to recognize the protein in infected erythrocytes both under IFA and immunoblotting (Fig.3.10A-E) whereas the anti-Band 3C (Fig. 3.10F-J) could recognize the protein, confirming the specificity of the antibodies for the Band 3 peptide.

IFA with anti-Band 3 (Fig.3.10K), and anti-Spectrin (Fig.3.11D) antibodies on PKH26 labelled *Babesia divergens*-iRBC, the lipid staining (Cy3) and immunostaining (Cy2) pattern both appeared rim like, delineating the parasite periphery akin to the PVM without overlapping the parasite cytoplasm.

Band 3 is a multiple membrane spanning high copy number protein of erythrocyte membrane and is involved in docking the cytoskeleton lying underneath the membrane and maintaining structural integrity in turn (Smith, 1987). However a significant fraction of the Band-3 protein in the erythrocyte is not cytoskeleton bound (Knowles et al., 1997). It has been argued to be a DRM associated protein as well (Murphy et al., 2004) and reportedly forms a macro complex together with interaction partners of integral and peripheral membrane proteins (Bruce et al., 2003). Association of different proteins with Band 3 itself had been attributed towards their presence on DRM fractions whereas association with cytoskeleton anchored- Band 3 had been attributed towards the inability of some other proteins to join DRM (Lauer et al., 2000, Bruce et al., 2003,

Murphy et al., 2004). Spectrin and Ankyrin free Band 3 reportedly has increased lateral mobility (Corbett et al., 1994b, Corbett et al., 1994a, Cho et al., 1998) and thus the ability to be associating with DRM fractions (Murphy et al., 2004). But absence of Band 3 or similar cytoskeleton associated proteins from the PVM however had so far been attributed towards it association with membrane cytoskeleton [Reviewed in (Lingelbach and Joiner, 1998)](Murphy et al., 2004)

Here I could show the presence of Spectrin itself in the newly formed PVM of *Babesia divergens*. It is a major cytoskeleton protein and had never been found on the PVM so far. Moreover any association with which had been reasoned for the exclusion of those associated proteins from PVM all along. By fluorescence assays with WGA I could also show the presence of sialic acid moieties of glycosylated protein and lipid components (of the erythrocyte membrane) recruited onto the *Babesia divergens*-PVM (Fig.3.13A-B) but not on the *P. falciparum*-PVM (Fig.3.16I-J) Though not distinguishable in my IFA (due to IgM antibody being incapable of entering through Triton-X generated smaller pores) under immuno-EM on similar batch of *B. divergens* -iRBC, another major integral membrane protein Glycophorin-A was also found localized onto its PVM (Repnik et al., 2015).

Glycophorin is the receptor well known to be involved during the invasion of *Plasmodium* and *Babesia* (Pasvol et al., 1982b, Cursino-Santos et al., 2014b, Lobo, 2005).Hence the presence of Glycophorin in the newly formed *B. divergens*-PVM is not surprising whereas its absence from the *P. falciparum*-PVM is. However, the incorporation of cytoskeletal protein Spectrin in *B. divergens*-PVM is astonishing.

Formation of PV is a fundamental cell biological phenomenon hence any probability of the *Babesia divergens* being random in the recruitment of the erythrocyte membrane proteins possibly be ruled out. Any bias in the methodology can also be ruled out because I could not detect internalized Band 3, Spectrin and/or Glycophorin in *Plasmodium falciparum* infected cells.

I repeated the experiments innumerable times and I went through this length only to establish my results against the previous publications those rationalized the exclusion of major erythrocyte membrane proteins (especially Band 3) and cytoskeletal proteins from the *Plasmodium*

falciparum-PVM to be a generic phenomenon owing to the organization and association of these proteins in erythrocyte cytoskeleton.

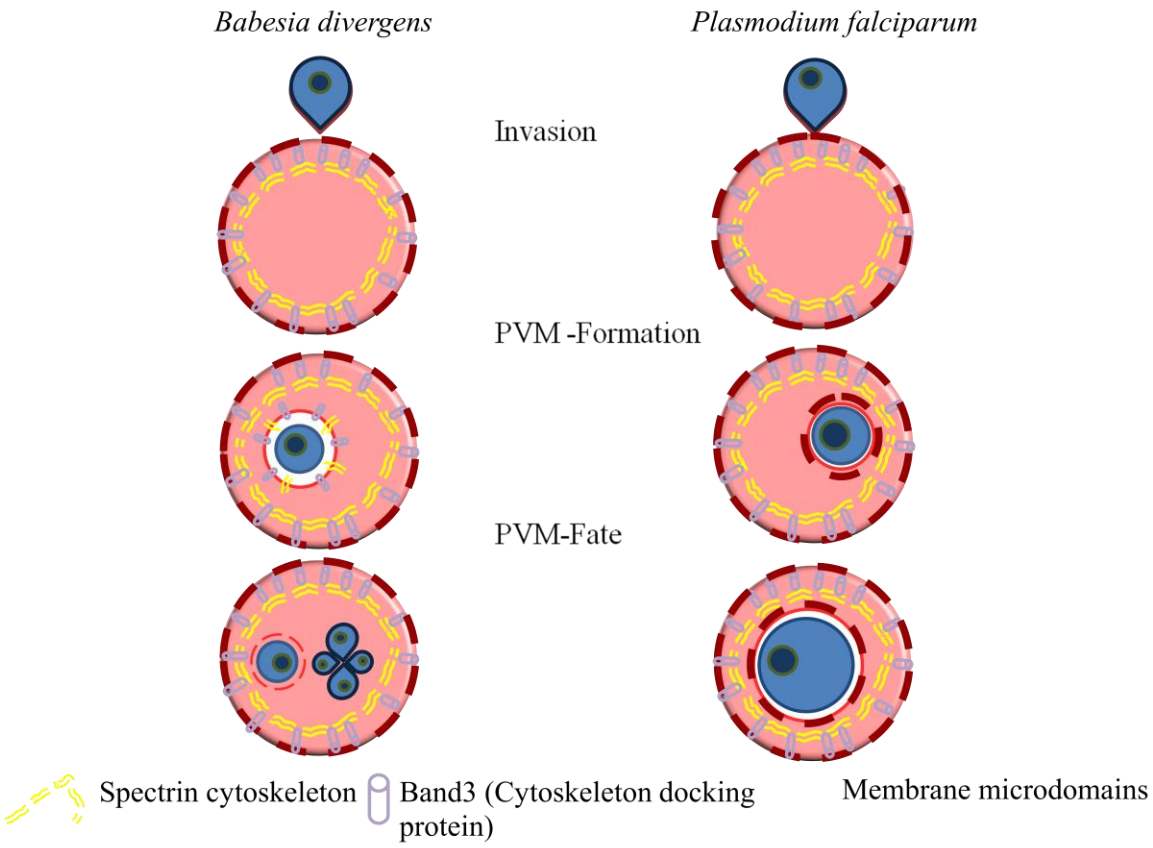


Figure 4.1 Differences in entry sites, parasitophorous vacuole membrane formation and disintegration in the of *Babesia divergens* and *Plasmodium falciparum*

The involvement and contribution of the erythrocyte seems to be different in the PVM formation in *P. falciparum* and *B. divergens*. In the PVM in *B .divergens* cytoskeletal proteins; cytoskeleton associated proteins and membrane glycoproteins was found whereas the invading *P. falciparum* doesn't seem to include these proteins onto their PVM. This can be implicating a preferred entry site for the *P. Falciparum* as was proposed by Murphy et al,(2004) as argued these are membrane microdomains responsible for the depletion of cytoskeletal proteins or in overall agility of the erythrocyte cytoskeleton at the site of entry. It is possible that *P. falciparum* retains the PVM possibly by active synthesis and transport of various proteins and lipid components to the PVM whereas *B. divergens* fails to do the same and the PVM disintegrates to not hinder any subsequent parasite growth.

My data indicate that there is no selective exclusion of erythrocyte cytoskeletal and cytoskeleton associated proteins from the PVM in *Babesia divergens*, an apicomplexan parasite closely related *Plasmodium falciparum*. Hence, my data argues against the rationale that such exclusion is a process governed by the inherent properties of the erythrocyte and advocates this to be a selective process on part of the parasite. It is more likely that the cytoskeletal and/or cytoskeleton

associated proteins are playing a varied role in the formation of PVM in these parasites and based on their role they are included or excluded (Fig.4.1).

However the presence or absence of specific proteins from the PVM of *P. falciparum* and/or *B. divergens* could also be a result of preferential entry site as was previously suggested by Murphy and colleagues (2004).

4.4 What novel does this project add to the repertoire of our understanding of red blood cell biology

Understandings about the contributors and the constituents of the PVM had undergone major changes over time. The earliest hypothesis based on the observations of different groups like McLaren and colleagues, (1977); Aikawa and colleagues, (1981); Atkinson and colleagues (1990) and Dluzewski and colleagues. (1989) was that the erythrocyte membrane proteins and lipids are altogether excluded from the developing PVM. (Bannister and Dluzewski, 1990, Dluzewski et al., 1989, McLaren et al., 1977, Aikawa et al., 1981, Atkinson and Aikawa, 1990).

These lead to the understandings that the substances stored in the apical complex (Rhoptries, Micronemes, dense granules) of these parasites are released into the erythrocyte membrane and thereafter these play crucial role in the formation of the PVM (Bannister and Dluzewski, 1990, Dluzewski et al., 1992). Experiments by Joiner and colleagues 1991 carried out in *Toxoplasma* spp. further advocated for the involvement of Rhoptries during the formation of the PVM in the Apicomplexans; as they proposed such an involvement gave the PVM a lipid composition different from that in the host cell plasma membrane, rendering it a more favourable for parasite survival (Joiner, 1991). Ward and colleagues in 1993 demonstrated that labelled lipid components from the erythrocyte surface were transferred to the parasite PVM and proposed that unlike erythrocyte membrane proteins (until this time) erythrocyte membrane lipids were actually being internalized and were recruited onto the newly formed PVM (Ward et al., 1993)(Ward 1993).

Emergence of the concept of cholesterol rich membrane microdomains in eukaryotic cells and erythrocytes alike (Lingwood and Simons, 2010) led to the re-examinations of the components of PVM in *P. falciparum*-iRBC (Murphy et al., 2004). Succeeding experiments over last decade had changed our understanding of the PVM-components. Findings by different groups have

shown recruitment of few erythrocyte membrane proteins like Flotillins and Aquaporins onto the PVM of *P.falciparum*.

Following their experiments in *Toxoplasma* spp. Mordue and colleagues (1999) proposed a membrane anchor dependant inclusion of erythrocyte membrane proteins in the newly formed PVM of *Toxoplasma* spp. and proposed inclusion for glycosylphosphatidylinositol (GPI) anchored proteins in the newly formed PVM (Mordue et al., 1999). Though the suitability of this model for all Apicomplexans was challenged when Duffy proteins and integrally associated membrane proteins like Flotillin-2 were shown recruited onto the PVM of *Plasmodium falciparum* (Lauer et al., 2000, Samuel et al., 2001). However capability of erythrocyte membrane proteins to associate and dissociate with the cholesterol rich microdomains had long been reasoned behind the inclusion or exclusion of these proteins from the newly forming PVM (Lauer et al., 2000)

The detergent mediated extraction of these cholesterol rich membrane microdomains as detergent resistant micro domain (DRM) units (arguably representing the raft fractions) had resulted into some ambiguity about the proteins associating with these DRM fractions (Salzer and Prohaska, 2001, Murphy et al., 2004, Samuel et al., 2001). However micro domain associated proteins like Flotillin 1 and 2, CD59 and Aquaporin-1 had been shown recruited onto the PVM of *P.falciparum* (Murphy et al., 2004).Later Aquaporin-3 was also found to be internalized and having undergone changes in its oligomeric structure (Bietz et al., 2009). Any bulk flow of high copy number proteins of the erythrocyte membrane to the PVM was yet not reported and the absence of these major erythrocyte membrane proteins from the newly formed PVM had so far been reasoned towards the biophysical properties of the erythrocyte and towards the varied association of these proteins with the erythrocyte cytoskeleton.(Lingelbach and Joiner, 1998, Murphy et al., 2004)

My study about the recruitment of erythrocyte membrane proteins onto the PVM of the Apicomplexan parasites provide a convincing evidence about recruitment of the membrane spanning-cytoskeleton associated protein Band 3 and of the cytoskeleton protein Spectrin onto the PVM of *Babesia divergens*, but not in *Plasmodium falciparum*. Additional observations in EM (Repnik et al., 2015) and internalization of WGA (in IFA) suggest that the erythrocyte

membrane glycolipids and/or glycoproteins like Glycophorins are also recruited onto the newly forming PVM of *Babesia divergens,* but not in *Plasmodium falciparum*.

The apparent difference in the recruitment of high copy number transmembrane proteins and/or cytoskeletal proteins between the two parasites is intriguing as it indicates the possibility of different modes of invasion for these two related parasites. The exclusion of cytoskeleton and cytoskeleton-associated proteins from the plasmodial PVM can be reflective of specific entry sites preferred by the *Plasmodium falciparum*, as was proposed by Murphy et al (2004) but which is seemingly not used by *Babesia divergens.* Membrane proteins have been shown to vary capacity to associate with these specific entry sites, which generally are cholesterol rich membrane microdomains (Lauer et al., 2000, Samuel et al., 2001, Salzer and Prohaska, 2001)

Additionally these domains are attributed for the ability to deplete the cytoskeleton proteins preferentially and/or affecting the elasticity of the cytoskeleton structure at the site of parasite entry (Murphy et al., 2007). These two parasites are known to interact with similar erythrocyte membrane receptors during invasion; amongst which Glycophorin and probably Band 3 are major ones (Cursino-Santos et al., 2014b, Lobo, 2005). Therefore the absence of these proteins in the Plasmodial-PVM but presence in the Babesial PVM is surprising.

Ultrastructure analysis of *Babesia microti* by Rudzinska in 1976 and on *Babesia divergens* by us in 2015 had demonstrated that the *Babesia* loses its PVM soon after infection. (Rudzinska, 1976, Repnik et al., 2015). My time course studies on PKH26 labelled-*Babesia divergens*-iRBC as well, showed a quick disintegration of babesial PVM. It was completed within the first few minutes after invasion. It is therefore obvious that the studies conducted on *Babesia* focuses on very early events during the PVM biogenesis. In comparison, the ring and trophozoite stages of *Plasmodium falciparum*, studied for similar analysis had focussed on these cells nearly 24hours and 48hours after invasion and PVM biogenesis.

P. falciparum is known to alter the PV environment greatly, by secreting and exporting integral membrane proteins and lipids into the PV (Gunther et al., 1991, Haldar et al., 2001, Johnson et al., 1994). Many of the secreted proteins are known proteases as well (Nyalwidhe and Lingelbach, 2006, Dowse et al., 2008). Therefore, it is possible that in the newly formed *Plasmodium falciparum*-PVM, the integral membrane proteins and cytoskeleton proteins were recruited; but thereafter they either were excluded or were degraded by the action of the parasite

proteases. A list of such candidate proteins had also been summerised by Blackman and colleagues in 2008 (Blackman, 2008)

In the IFA experiments *Plasmodium falciparum* and *Babesia divergens* both were found to recruit some proteins onto their PVM those are recovered in the DRM and non-DRM fractions of membranes in non-infected erythrocytes. Nevertheless, more quantitative analyses are required to investigate if these DRM-associated internalized components were indeed DRM associated at the time of invasion. Parallaly to understand the recruitment of cytoskeletal or cytoskeleton associated proteins in the newly formed PVM of *Plasmodium falciparum*, more work on early stages of the parasite should be conducted.

Nevertheless, in totality my data argues against the hypothesis in practice, about the exclusion of the high copy number transmembrane proteins and/or cytoskeletal proteins of erythrocyte, owing to the biophysical properties of the erythrocyte and organisation of these proteins in the membrane. Parallaly my data also argues against the fact that biophysical properties of the erythrocyte membrane should lead the parasites to preferentially select specific domains within the membrane for continuing on with invasion. Instead, in advocates for the possibility of a difference in entry sites for these two parasites and a difference in the involvement of RBC cytoskeleton and/or cytoskeleton associated proteins in the formation of PVM in these two apicomplexan parasites altogether.

5 Outlook

By studying parasite infected human erythrocyte we have been able to unravel inducible properties, generally silent in these quiescent cells and cell biological properties unseen in non-infected erythrocytes. In the complete absence of any genetic programme and limited metabolic activities, accomplishing such major alterations on part of the erythrocyte remains an enigma.

It is more seemingly that these alterations are driven, to a great extent, by the parasite and therefore it implies a major overhauling of the intra-erythrocytic milieu by the proteins coaded by the parasite. *Plasmodium falciparum* is known to transcribe and export few hundreds of proteins to various intracellular compartments in order to modulate the activities in infected erythrocytes (Sargeant et al., 2006). *Babaesia* is known to alter the host cell similar in line to done by Plasmodium but mostly to a lesser extent.

None the less, to identify specific alterations of the erythrocyte done by the parasites and the moderators behind such alterations, the *Babesia* exportome should be determined.

Another interesting work will be to identify if there is any formation of Tight or moving junction during the invasion of *Babesia divergens* and identify the erythrocyte and parasite components of the tight junction contributing ultimately towards the invasion.

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Curriculum Vitae

Personal data

Name: Preetish Gangopadhyay

Date of birth: 15th September 1983

Place of birth: Calcutta, West Bengal, India

University education

January 2012- 2015

Preparation of doctoral thesis in the research group of Prof. Dr. Klaus Lingelbach

Philipps-University of Marburg, Department of Parasitology, Faculty of Biology

Title of dissertation: "Recruitment of erythrocyte membrane components by apicomplexan parasites *Plasmodium falciparum* and *Babesia divergens*"

October 2010-September 2011

Master of Science by Research in Functional Genomics,

Faculty of Biology, University of York, York, United Kingdom

Master thesis at Cancer Research UK, Yorkshire Unit, University of York, UK

August 2005-September 2007

Master of Science in Biotechnology

Faculty of Biotechnology and Microbiology, Bangalore University, Bangalore, Karnataka, India

Master thesis: at Department of Immunology and Molecular biology; AMC College, Bangalore, India.

July 2002-August 2005

Bachelor of Science in Biotechnology

Faculty of Biotechnology and Microbiology, Bangalore University, Bangalore, Karnataka, India

Bachelor thesis: at Department of Molecular biology Best Biotech research laboratories, Bangalore, India.

School education:

1986-2002

Jadavpur Vidyapith, under West Bengal board of Secondary and higher secondary education

ERKLÄRUNG

ich versichere, dass ich meine Dissertation

The recruitment of erythrocyte membrane components by obligate intracellular parasites: *Babesia divergens* and *Plasmodium falciparum*

selbstständig, ohne unerlaubte Hilfe angefertigt und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe.

Die Dissertation wurde in der jetzigen oder einer ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

Marburg, den __.05.2015 _____

(Ort/Datum) (Preetish Gangopadhyay)